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PLANT PHYSIOLOGY

VOLUME 28 APRIL, 1953 NUMBER 2

EFFECT OF QUALITY AND INTENSITY OF SOLAR RADIATION ON INJURY OF LEMON FRUIT BY SULPHUR TREATMENT IN THE FIELD[1]

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Received April 25, 1952

Introduction

For many years it has been recognized that annual and perennial plants of either ornamental or crop type, when sprayed with lime-sulphur or oil-sulphur or dusted with elemental sulphur for control of insects or fungi, may be injured during periods of hot weather (**6, 8, 9, 11, 14, 16, 21, 23, 26, 27, 28, 30, 31, 32, 35, 36, 37, 38, 44, 49, 50, 52**). Certain crops, and even certain varieties of a given species, are more sensitive to sulphur burn than others, regardless of temperature (**36, 48**).

Sunburn of agricultural crops has received attention by HARVEY (**18**), MACGILLIVRAY (**25**), and LORENZ and KNOTT (**24**), but as far as the authors are aware, no published work has dealt with the physical factors attending hot weather which effect sulphur burn. Although the factors responsible for sunburn and for sulphur burn may be different, the distinction has not been clear (**28**). An understanding of these factors is essential to the adoption of preventive measures or to the development of safer sulphur dusts and sprays.

The present paper reports a field experiment conducted in 1942 and involving 65 lemon trees and 19,009 fruits. The experiment was designed to show the effect of reduction in intensity of total solar radiation, and of reduction of ultraviolet intensity in solar radiation on sunburn and sulphur burn of lemon fruit.

Materials and methods

To determine the principal environmental factors involved in the injury of lemon fruit by sulphur treatment in hot weather (fig. 1), lemon trees ranging in age from 2 to 25 years, in a grove composed of Lisbon and Eureka lemon scions on Rough lemon rootstock, were chosen at random by drawing numbers. Six to 10 trees were used in each of eight treatments.

[1] Paper no. 751, University of California Citrus Experiment Station, Riverside, California.

Trees of three lots were dusted with 97.5% 325-mesh pure sulphur dust (2.5% inert ingredient). Each tree was dusted with a portable power duster under a fumigation tent, to prevent spreading the dust to other trees. The tent was removed when the dust had settled, and the trees so dusted were treated in three different ways: Each tree of the first lot was covered on top and sides with cheesecloth (No. 50 Curity) tacked to a frame constructed of two-inch by four-inch wood beams, as shown in figure 2 A and B. Such covering is referred to hereafter as a tent. Trees of the second lot were not covered with cheesecloth. On trees of the third lot, the fruits exposed to direct rays of the sun were covered with sunshades of yellow cellulose acetate sheeting (ultraviolet filters) fastened over the fruit pedicels with florist clips, as shown in figure 3.

Three other lots of trees (the fourth, fifth, and sixth) were not dusted with sulphur. Trees of the fourth lot were covered with cheesecloth tents

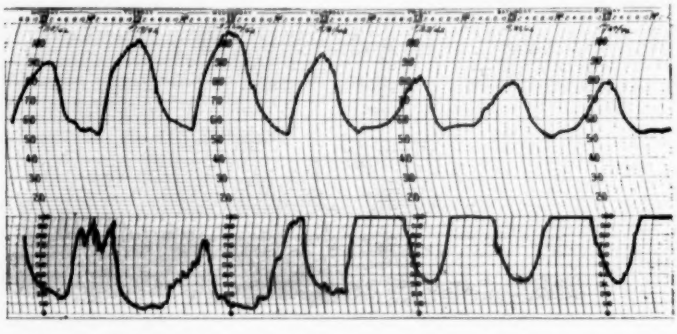

FIG. 1. Hygrothermographic chart for week of May 18–24, 1942, at Corona, California, showing air temperatures in the shade (upper curve) and relative humidity (lower curve).

(fig. 2 A). Sun-exposed fruits of trees of the fifth lot were covered with sunshades (ultraviolet filters) of yellow cellulose sheeting (fig. 3). Trees of the sixth lot, which were neither dusted with sulphur nor covered, served as controls. The random distribution of the cheesecloth-covered trees in the 2.5-acre grove is shown in figure 2 C. A set of six trees was chosen to be treated, treated and covered, and covered in one day. Prior to the first hot period and between May 9 and 19, 59 trees had been placed under experimental control. Prior to the second hot period and between June 3 and 6, an additional set of six trees was placed under experimental control.

Light-transmission measurements made by the use of a photoelectric cell showed that the cheesecloth transmitted 73.1 to 73.7% of the light from a Mazda lamp. The cellulose acetate sheeting 0.135 mm. thick used for fruit sunshades transmitted 75 to 92% of the visible spectrum for wave lengths of 550 to 700 mμ, 2% for wave lengths of 350 to 500 mμ, and none for those of 275 to 350 mμ. This sheeting cut out (absorbed) the ultraviolet radiation and is therefore referred to herein as an ultraviolet filter. The visible,

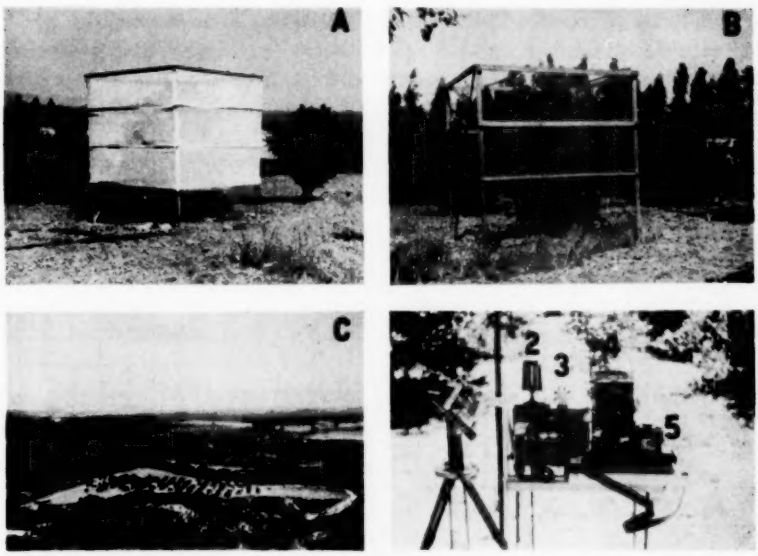

FIG. 2. A. Completed tent with cheesecloth strips 36 inches wide tacked to supporting frame. B. Wooden supporting structure for cheesecloth tent over small lemon tree. C. Experimental lemon grove (2.5 acres) near Corona, California, showing random distribution of trees covered with cheesecloth tents. D. Apparatus used in measuring meteorological factors and fruit temperatures at site of experiment: 1. Eppley thermopile (mounted on sun site) for measuring solar radiation; 2. Tycos hygrodeik; 3. Taylor anemometer; 4. Insulated vacuum-bottle ice bath for thermocouple cold junctions, fitted with switch panel and 18-guage copper-constantan thermocouples, top, stirrer, and thermometer; 5. Leeds-Northrup K-2 potentiometer; 6. No. 2420 galvanometer, dry cells, storage battery, and enclosed standard cell.

infrared, and ultraviolet light-transmission measurements (figs. 4 A, B and D) were made with Beckman spectrophotometers.

In a seventh and eighth lot of trees consisting, respectively, of nine old trees and eight very young trees, the direct effects of light absorption by the fruit were eliminated by the use of a heavy spray of lime-sulphur, which has a high degree of visible-light reflectivity. This spray was composed of 98

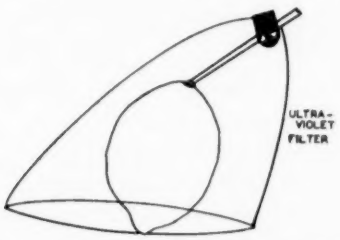

FIG. 3. Diagram showing method of attaching yellow cellulose acetate sheeting over fruit pedicel with florist's clip to form ultraviolet filter.

gallons of water, two gallons of lime-sulphur solution, two pounds of elemental dusting sulphur, two pounds of caseinate spreader, and two pounds of 23% zinc sulphate, and was applied at the rate of 5 to 10 gallons per tree, the amount depending on the tree size. Zinc sulphate was omitted from the spray applied to two trees.

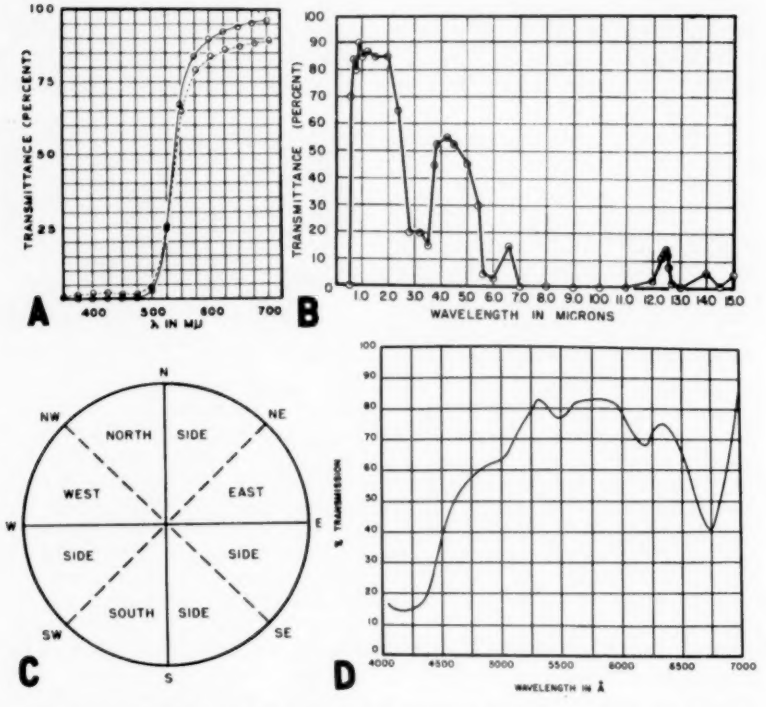

FIG. 4. A. Visible spectrum transmission curves of dry (broken line) and wet (continuous line) yellow cellulose acetate sheeting. No transmission was recorded in the ultraviolet spectrum from 350 mμ down to 275 mμ. Sunlight radiation in ultraviolet begins at 290 mμ. B. Infrared spectrum transmission curve of yellow cellulose acetate sheeting. Transmission at 0.7 μ (80 to 85%) and at 0.5 μ (zero) corresponds roughly with that shown at 700 mμ and at 500 mμ in A. C. Diagram illustrating methods of laying out ground beneath tree to indicate tree sides (between broken lines) for precision counting of fruit on tree. D. Light transmission of surface section 0.79 mm. thick of green lemon peel in percentage of 100% transmission of similar section of yellow peel.

Meteorological and fruit-temperature measurements were made at the site of the experiment (fig. 2 D). For counting the fruit on the tree, the ground beneath the tree was laid out in quadrants, using a compass as shown in figure 4 C. Slats marked the first divisional line. Quadrant halves from NW to NE represented the north side of the tree, NE to SE the east side, etc. A string stretched around the tree divided it into top and bottom halves.

One of two observers called the burned and unburned fruits individually, while the second, using two Veeder bond tallies, recorded the number in each category for each quadrant in the top and bottom portions of each tree.

Tree height was determined from TURRELL's tables (**39**) from percentage inclination measured with a Plummer hypsometer, and tree volume was determined from LINDGREN's tables (**22**). Pertinent characteristics of the trees and of the treatments applied are presented in table I. Trees used for the first hot period were not used for the second hot period.

Throughout the preparation period and during the experiment, normal cultural practices were maintained in the grove. All ripe yellow lemons were picked before preparation for the experiment began. A heavy rain on April 11 was followed by light rains until April 28. Low sprinkler irrigation

TABLE I

EXPERIMENTAL TREE TREATMENT AND CHARACTERISTICS.

Date	Treatment	Total number of trees	Age of trees	Tree volume	Total number of fruits
			yrs.	*cu. ft.*	
May 19–20, 1942 (first hot period)	Sulphur dust	8	4, 5, 25	525–550	2,826
	Lime-sulphur spray (old trees)	9	10	1400–1600	5,662
	Lime-sulphur spray (young trees)	8	2	10–15	46
	Sulphur dust and tent	8	4, 5	500–650	2,532
	Sulphur dust and ultraviolet filters	6	4, 5, 25	525–725	1,984
	Tent only	8	4, 5	400–600	2,021
	Ultraviolet filters only	6	4, 5	475–675	1,573
	None (control)	6	4, 5	475–675	1,063
	Total	59			17,707
July 4–10, 1942 (second hot period)	Sulphur dust	2	4, 5	500–650	434
	Sulphur dust and tent	2	4, 5	225–375	611
	None (control)	2	4, 5	500–650	257
	Total	6			1,302

was employed at two-week intervals after April 28, until May 21, when furrow irrigation was used (from May 28 to June 5, and from June 23 to July 3) to supplement the sprinklers. Thus there was adequate soil moisture during the hot periods.

METEOROLOGY

During the period from April 1 to May 1, 1942, while the cheesecloth tents were being constructed and ultraviolet filters were being applied, weather conditions were moderate, maximum daily air temperatures ranging from a low of 60° to a high of 86° F. Sulphur dust and lime-sulphur spray was applied to the various trees from May 10 to May 18. The first eighteen days in May were also marked by moderate air temperatures and bright sunshine (637 to 649 gm. cal./cm.^{2}/day). During this period, maximum

TABLE II

METEOROLOGICAL DATA FOR TWO HOT PERIODS, MAY 19–20 AND JULY 4–10, 1942, AT RIVERSIDE, AND FOR DAYS PRIOR TO AND FOLLOWING THESE PERIODS (PACIFIC STANDARD TIME).

Date	Total solar radiation	Relative humidity**	Minimum water-vapor density	Maximum air temperature	Hours above 100° F	Prevailing wind direction†			Average wind velocity		
						6:00 A.M. to 8:00 A.M.	8:00 A.M. to 10:00 A.M.	10:00 A.M. to 4:00 P.M.	6:00 A.M. to 8:00 A.M.	8:00 A.M. to 10:00 A.M.	10:00 A.M. to 4:00 P.M.
	gm. cal./ cm.²/day	%	*grains/ cu. ft.*	°F					*mi./hr.*	*mi./hr.*	*mi./hr.*
May 16	637	32	2.994	75	0	S	W	W	2	3.8	6.3
17	671	31	3.186	78	0	S	W	W	1	3.5	6.0
18	649	16	2.165	87	0	E	W	W	1	3.5	6.5
19*	629	4	0.885	104	2.5	S	S → N	N → NW	1.5	2.0	6.0
20*	634	6	1.483	108	6	N	N	N → SW	< 1	< 2.0	7.3
21	559	18	2.995	94	0	SW	W	W	1.5	2.7	7.2
22	489	30	3.596	83	0	W	W	W	1.5	2.3	6.2
23	611	32	3.392	79	0	SE	SW	W	4.5	3.8	5.7
24	456	31	3.286	79	0	SE	W	W	< 1	2.0	4.2
July 1	623	18	3.361	98	0	SW	SW	SW.W	< 1	3.0	6.5
2	623	20	3.734	98	0	SW	W	SW.W	< 1	3.0	8.0
3	645	28	4.658	94	0	SW	SW	SW.W	2.0	2.6	6.6
4*	670	6	1.328	104	2	W	NW	SW	4.0	2.2	6.0
5*	668	4	0.989	108	6.5	N	NW	NW → SW	< 1	2.3	7.9
6*	648	8	2.033	109	6.5	SW	SW	NW → SW	0.2	2.5	7.1
7*	633	8	2.089	110	6.5	NW	NW	SW	0	2.0	7.0
8*	670	10	2.339	106	6.0	NW	NW	NW	< 1	3.0	6.6
9*	670	29	5.732	100	0.5	NW	NW	NW → SW	1.5	3.0	8.5
10*	685	14	2.929	102	0.5	NW	SW	SW	< 1	3.0	6.5
11	661	29	4.966	95	0	NW	NW	SW	< 1	3.5	7.1
12	652	35	6.350	97	0	NW	NW	SW	2	2.7	6.6

*Hot days.
**Recorded in open in dry area.
†Arrow indicates changing direction during period.

daily air temperatures ranged from a low of 68° to a high of 91° F, while sunlight intensity ranged generally from 77 to 85 gram calories per cm.^{2} from 12 M. to 1 P.M. and averaged about 80 gram calories for this time interval.

A light westerly wind (ocean air) prevailing from May 16 to May 18 shifted to the north (desert air) on May 19, and though the total solar radiation dropped to 629 gm. cal./cm.^{2}/day (77.5 for noon hour), the usual midday relative humidity of 30% dropped to approximately 5% (fig. 1); the midday temperature rose to 100° F by 11:30 A.M. and continued to rise until it reached a maximum of 104° F at 12:30 P.M., remaining above 100° F until 2:00 P.M. (total two and one half hours). On May 20 the solar radiation rose to 634 gm. cal./cm.^{2}/day (78.0 for noon hour), the air temperature passed 100° F at 9:30 A.M., reached 108° at 11:45, and remained above 100° for approximately six hours, in which period the relative humidity dropped to approximately 8% (fig. 1). On the following days the air temperature moderated, and the relative humidity rose to approximately the usual percentage.

For the first three days in July, temperatures ranged from 94° to 98° F, total solar radiation ranged from 623 to 648 gm. cal./cm.^{2}/day, and relative humidity ranged from 18 to 28%, with a southwest breeze (ocean air). On July 4 the prevailing wind shifted to the northwest, driving the desert air into the Santa Ana Valley, relative humidity dropped to 6%, solar radiation increased to 670 gm. cal./cm.^{2}/day, and the midday temperature remained at 104° F for two hours. On July 5, 6, 7, 8, 9, and 10, midday temperatures of 100° to 110° F were recorded. Meteorological data for the two hot periods are shown in table II. Sulphur dust had been applied to the various trees on June 4 and 5.

Results

Fruit injury

Little fruit burn was observed on May 19, the beginning of the first hot period, but by noon of May 20 considerable burn had developed. Since temperatures below 100° F were predicted for May 21, counting was begun on that date and completed within the following week of relatively cool weather. During the ensuing cool period, preparations were made for a second trial of sulphur burn. Four trees were sulphur-dusted, and two of these trees were subsequently covered with tents. Two untreated trees were selected to serve as controls.

In the second hot period as in the first hot period, both sunburn and sulphur burn of fruit were evident on the second day. Counts of injured fruit resulting from the second hot period were delayed until after moderate weather again prevailed (July 11). No fruits were burned during either hot period, except those exposed to direct solar radiation, and little leaf burn was observed except on the trees treated with lime-sulphur and zinc-sulphate spray.

Results of counts of burned and unburned fruit for both hot periods were combined and are shown in percentages in table III. Untreated trees covered

with cheesecloth tents had no injured fruit, but on untreated trees not under tents 0.15% of the fruit was injured. Although this difference is only significant for P = 0.2 (table VIII), it indicates that a small number of untreated fruits sunburned, and that reduction of sunlight intensity prevented injury. On those nondusted trees on which fruits in exposed positions were covered with ultraviolet filters, 1.07% of the fruit was injured, as compared with 0.15% for the nondusted controls (table III). This is significant at the 5% level (table VIII) and indicates that the ultraviolet filters did not reduce injury from sunlight but significantly increased the injury over that suffered by uncovered fruit.

Injury of sulphur-dusted fruit shielded by ultraviolet filters (10.54%) was a little higher than that of unprotected sulphur-dusted fruit (9.88%) (table III), but not significantly so (table VIII), and this suggests that

TABLE III

EFFECT OF VARIOUS TREATMENTS ON INJURY TO LEMON FRUITS BY SUN RADIATION DURING TWO HOT PERIODS, MAY 19–20 AND JULY 4–10, 1942 (DATA COMBINED).

Treatment	Number of trees treated	Total number of fruits	Fruits injured	
			no.	%
Sulphur dust	10	3,260	322	9.88
Lime-sulphur spray (old trees)	9	5,662	965	17.04
Lime-sulphur spray (young trees)	8	46	18	34.72
Sulphur dust and tent	10	3,143	140	3.58
Sulphur dust and ultraviolet filters	6	1,984	240	10.54
Mean	8.6	2,819	337	15.14
Tent	8	2,021	0	0.0
Ultraviolet filters	6	1,573	15	1.07
None (control)	8	1,320	2	0.15
Mean	7.33	1,638	5.67	0.41

although the filters increased sunburn, they were not effective in increasing or reducing sulphur burn. Elimination of ultraviolet radiation from total sun radiation, therefore, did not protect against either sunburn or sulphur burn.

On sulphur-dusted trees not covered by cheesecloth tents 9.88% of the fruit was injured, whereas on similarly treated trees covered with cheesecloth tents only 3.58% of the fruit was injured (table III). The difference is significant at the 10% level (table VIII). Thus the reduction of solar radiation in the visible region by 26 to 27% reduces the amount of injury from sulphur dust. It is clear, also, that sulphur dust increases the amount of injury resulting from the effects of direct solar radiation, since 9.88% of the uncovered, sulphur-dusted fruit was burned, as compared with 0.15% of that of the nondusted, uncovered control (table III). This difference is significant at the 1% level (table VIII).

Trees sprayed with lime-sulphur had somewhat more fruit injury than

sulphur-dusted trees (table III), the difference being significant at approximately the 10% level (table VIII). On old trees sprayed with lime-sulphur, 17.04% of the fruit was injured; on young trees, 34.72% of the fruit was injured (table III). The difference in percentage injury of fruit of young and old trees is significant at the 5% level (table VIII). Burned fruit on the two 25-year-old trees sprayed with lime-sulphur without zinc sulphate averaged 2.6 and 14.3%, respectively, or slightly less than on the lime-sulphur-zinc-sprayed trees.

TABLE IV

EFFECT OF FRUIT LOCATION ON TREE (SIDES) ON PROPORTION OF TOTAL LEMON FRUITS AND OF SUNBURNED AND/OR SULPHUR-BURNED FRUITS ON EACH SIDE.

Treatment	North side		East side		South side		West side	
	Per cent. of total fruit	Per cent. burned fruit	Per cent. of total fruit	Per cent. burned fruit	Per cent. of total fruit	Per cent. burned fruit	Per cent. of total fruit	Per cent. burned fruit
Sulphur dust	24.14	1.78	23.40	2.73	27.58	3.59	24.88	1.87
Lime-sulphur spray	23.05	2.68	24.53	5.51	26.26	6.34	26.16	2.76
Sulphur dust and tent	23.42	0.86	25.04	1.46	27.39	1.37	24.15	0.76
Sulphur dust and ultra-violet filters	27.52	2.52	21.27	3.18	26.71	4.74	24.50	1.66
Mean	24.53	1.96	23.56	3.22	26.99	4.01	24.92	1.76
Tent	24.44	0.0	24.84	0.0	24.99	0.0	25.73	0.0
Ultraviolet filters	24.48	0.0	21.04	0.25	26.81	0.57	25.87	0.13
Control	24.32	0.0	21.29	0.08	28.74	0.0	25.45	0.08
Mean	24.41	0.0	22.39	0.11	26.85	0.19	25.68	0.07
Mean (all treatments) and standard deviation	24.48 ± 1.34	1.12	23.06 ± 1.68	1.89	26.95 ± 1.16	2.37	25.25 ± 0.696	1.04

On all sulphur-treated trees except those covered by tents, the percentages of sulphur-burned and sunburned fruit were greatest on the south sides of the trees (table IV). Comparison of sulphur injury on the south sides of sulphur-treated trees with that on the north sides shows a difference that is significant at the 5% level (table VIII). The control trees showed a small and nonsignificant amount of sunburn on east and west sides (table IV).

It might be argued that the largest proportion of fruit is produced on the south side of the tree, and that, since both the sun angle and the sun's east-to-west progression across the south horizon increases the intensity and duration of radiation on the south sides of the trees, the highest proportion

of burned fruit might be expected there. As shown in table IV, higher average percentages of fruits were borne on the south sides of the trees than on any of the other sides. The east sides of the trees bore the smallest average percentage of fruits, and the t test shows that the difference between the number of fruits borne on the east and north sides is highly significant (table VIII). The t tests based on standard deviations of means show that the differences between the number of fruits borne on the south side and on the north or west sides are also significantly different.

The intensity of solar radiation and its duration might be expected to be greater on the exposed tops of the trees than on the bottoms. Separate counts of fruit on the top halves of trees, as compared with those on the bottom halves, showed that the higher percentages of sulphur-burned fruit were commonly found on the top halves (table V). Analysis of variance

TABLE V

EFFECT OF FRUIT LOCATION ON TREE (TOP OR BOTTOM HALVES) ON PERCENTAGE OF SUNBURNED AND SULPHUR-BURNED LEMONS (BASED ON TOTAL NUMBER OF FRUITS ON TREE).

Treatment	Burned fruit on tree halves	
	Bottom	Top
	%	%
Sulphur dust	3.99	5.89
Lime-sulphur spray	7.14	10.17
Sulphur dust and tent	0.22	4.23
Sulphur dust and ultraviolet filters	3.73	8.37
Mean	3.77	7.17
Tent	0.00	0.00
Ultraviolet filters	0.84	0.13
Control	0.08	0.08
Mean	0.31	0.07
Mean, all treatments	2.29	4.12

shows that the difference in the percentages of burned fruit on the top and bottom halves of sulphur-dusted lemon trees is significantly different at the 5% level (table VIII). For the various other sulphur treatments (lime-sulphur spray, sulphur dust and tent, and sulphur dust and ultraviolet filters), the differences between the means (tree tops *vs.* bottoms) suggest that they also are significantly different. The effect of sulphur in increasing burn of top fruit is especially striking (table V) and significant (table VIII).

The relative light transmission of sections 0.79 mm. thick from the peel of green and yellow fruits was measured to determine the magnitude of the effect of peel color on light absorption. The transmission of the green peel (fig. 4 D) is shown in percentage of 100% transmission of yellow peel. Integration of this curve gives 66.5% as the average transmission of green peel, as compared with that of yellow peel. Thus green peel absorbs about one third more energy than yellow peel, without accounting for the quanta differences in the spectral regions covered.

TABLE VI

EFFECT OF COLOR AND SIZE OF LEMONS ON THE PERCENTAGE OF SUNBURNED AND SULPHUR-BURNED FRUIT (BASED ON TOTAL NUMBER OF FRUITS OF EACH KIND IN EACH TREATMENT).

Treatment	Per cent. burned fruit			
	Small green	Large green	Small yellow	Large yellow
Sulphur dust	9.3	10.5	33.3	6.3
Lime-sulphur spray	14.4	18.8	44.5	23.7
Sulphur dust and tent	3.4	4.6	33.3	6.6
Sulphur dust and ultraviolet filters	10.0	14.3	11.1	4.3
Mean	9.3	12.1	30.6	10.2
Tent only	0.0	0.0	....	0.0
Ultraviolet filters	1.0	0.9	0.0	1.3
Control	0.0	0.3	0.0	0.0
Mean	0.3	0.4	0.0	0.4
Mean, all treatments	5.4	7.1	20.4	6.0

The effects of size and color of fruit on percentages of sunburn and sulphur-burn are evaluated in table VI, where a higher percentage of sulphur burn is shown to have occurred in small yellow fruit in all treatments except those employing ultraviolet filters. The number of yellow fruits involved was small, however (42 for all experiments). For the fruit treated with lime-sulphur, analysis of variance of percentages of sulphur burn of small green, large green, small yellow, and large yellow fruit as groups indicated no significant differences (table VIII). Analysis of variance of percentages of sulphur burn of large green fruit and large yellow fruit under this treatment likewise revealed no significant difference (table VIII). The percentage of lime-sulphur burn was greater for large green fruit than for small green fruit, but since the difference was significant only at the 20% level (table VIII), little confidence should be placed in this finding. For the sulphur-dusted fruit, analysis of variance indicated no significant differences in the percentages of sulphur-burned fruit when the comparisons were made between large green fruit and small green fruit or between large green fruit and large yellow fruit (table VIII).

TABLE VII

EFFECT OF SULPHUR DUST ON PERCENTAGE OF SULPHUR-BURNED LEMONS (OF TOTAL NUMBER OF FRUITS ON TREES) IN TWO DIFFERENT PERIODS OF HOT WEATHER, 1942.

Treatment	Burned fruit	
	First hot period (May 19–20)	Second hot period (July 4–10)
	%	%
Sulphur dust	9.26	8.35
Sulphur dust and tent	4.48	0.00
None (control)	0.00	0.78
Mean	4.58	3.04

TABLE VIII

ANALYSIS OF VARIANCE, WITH F AND *t* VALUES FOR SUNBURN AND/OR SULPHUR-BURN COMPARISONS FOR VARIOUS TREATMENTS OF LEMON TREES AND FRUIT.

Table	Comparison	Between groups		Within groups		F		*t*	
		Degrees of freedom	Mean square	Degrees of freedom	Mean square	Found	For 5% level significance	Found	Required for significance
III	Tent only *vs.* control	1	0.14	14	0.063	2.24	4.60	1.49*	1.34
	U.V. filters *vs.* control	1	1.80	12	0.32	5.63†	4.75	....	....
	Sulphur dust and ultra-violet filters *vs.* sulphur dust	1	5.57	12	38.85	6.97	243.91	....	....
	Sulphur dust and tent *vs.* sulphur dust	1	91.68	14	23.78	3.86	4.60	1.96**	1.75
	Lime-sulphur spray *vs.* sulphur dust	1	139.12	14	47.60	2.92	4.60	1.71**	1.75
	Sulphur dust *vs.* control	1	329.42	14	15.98	20.61††	4.60	....	....
	Lime-sulphur (old trees) *vs.* lime-sulphur (young trees)	1	1531.15	14	310.55	4.93†	4.60	....	....
IV	South sides of trees *vs.* north sides of trees (all sulphur-treated)	1	220.58	18	46.61	4.73†	4.41	....	....
	No. fruits on south sides *vs.* no. fruits on east sides (all treatments)	....	....	....	....	....	....	5.11**	3.01
V	Top halves of trees *vs.* bottom halves (all sulphur-dusted)	1	437.67	18	59.05	7.41†	4.41	....	....
VI	Small green fruit *vs.* large green fruit *vs.* small yellow fruit *vs.* large yellow fruit (all lime-sulphur-sprayed)	3	549.12	26	600.18	1.09	8.53	....	....
	Large green fruit *vs.* large yellow fruit (all lime-sulphur-sprayed)	1	26.35	14	135.09	5.13	243.91	....	....
	Small green fruit *vs.* large green fruit (all lime-sulphur-sprayed)	1	138.59	14	58.61	2.36	4.60	1.54*	1.34
	Small green fruit *vs.* large green fruit (all sulphur-dusted)	1	2.36	14	36.65	15.53	243.91	....	....
	Large green fruit *vs.* large yellow fruit (all sulphur-dusted)	1	2.04	14	172.74	84.68	243.91	....	....
VII	Fruit of May hot period *vs.* fruit of July hot period								
	Sulphur dusted	1	1.34	8	31.92	23.82	238.89	...	....
	Sulphur-dusted *vs.* sulphur-dusted and tent	1	23.26	18	29.25	1.26	243.91	0.89	1.33
	Control	1	0.008	6	0.026	3.14	233.97	....	....

*Significant at P = 0.2.
**Significant at P = 0.1.
†Significant at 5% level.
††Significant at 1% level.

Growers of citrus in California have generally observed a higher percentage of sulphur burn in spring than in summer. The spring hot period infrequently occurs in April with temperatures as high as 113° F, when the foliage produced during the March flush of growth is still immature. In the present experiments seasonal effects have been minimized because the first hot period occurred in the middle of May, when the leaves of the spring flush of growth had matured and the second and smaller flush was just beginning. The effects of sulphur dust on sulphur burn of fruit for the May hot period and for the July hot period are shown in table VII. There was a smaller percentage of sulphur burn in July than in May, both for trees under tents and for those completely exposed. No sunburn occurred in May; 0.78% was recorded for July. This suggests that susceptibility to sulphur burn is a characteristic of fruit in the spring or that the reduction of burn in summer

TABLE IX

PEEL-SURFACE TEMPERATURE OF LEMONS ON TREE ON TWO DIFFERENT DAYS, UNDER VARIOUS EXPERIMENTAL CONDITIONS.

Date		Peel temperature of fruit					Air temperature in shade
		Under ultraviolet filters in sun	Inside tent		Outside tent		
			In sun	In shade	In sun	In shade	
		°F	°F	°F	°F	°F	°F
July 3, 1942*	1	119.0	95.6	86.8	96.9	84.2	89.0
	2	119.2	93.4	84.0	92.4	83.2	89.0
	3	....	96.3	87.9	98.7	83.5	90.0
	4	....	101.8	85.1	111.0	86.5	....
	Mean	119.1	96.8	85.9	99.7	84.3	89.3
July 6, 1942**	1	113.8	102.0	98.0	113.8	99.8	102
	2	113.6	103.8	99.1	115.6	98.1	106
	3	111.7	108.2	....	111.5	98.0	....
	4	113.5	....	....	....	....	....
	Mean	113.1	104.6	98.5	113.6	98.6	104

*NE wind velocity, 2.5 mi./hr.; relative humidity, 42–43.5%.
**NW wind velocity, 5.68 mi./hr.; relative humidity, 16–19%.

may result from the fact that exposed fruits are removed from the tree by natural causes before this time, or are shaded by newly developed leaves. These results (table VII), except the sunburn data for the control, are in accord with field observations, but the results could be entirely fortuitous, as indicated by analyses of variance (table VIII).

Temperature of fruit peel

To find out, if possible, why both sulphur-dusted and nondusted lemons burned badly under ultraviolet filters, while fruit shaded with cheesecloth did not, peel-surface temperatures of fruits were measured both in shade and in direct sunlight when (a) covered with cheesecloth tents, (b) covered with ultraviolet filters, and (c) without covers.

Effect of cheesecloth tents.—In general, peel temperatures of fruit in the sun in cheesecloth tents were from 2.2° cooler to 11.8° warmer than

air temperature in the shade, while peel temperatures of shaded fruit in cheesecloth tents were from 2.1 to 6.9° cooler than air temperature in the shade (table IX). Peel temperatures of fruit in the sun outside the tents were somewhat warmer than those of fruit in the sun inside the tents, ranging, in one case, from 1.0° cooler to 11.8° warmer than those of fruit in the sun inside the tents, while peel temperatures of unprotected fruit in shade averaged from 5.0 to 5.4° cooler than air temperature (table IX), or about the same as those of shaded fruit inside tents. Air temperatures in shade inside tents (table X) were 1 to 2° higher than air temperatures in the shade in the open. Although fruits in direct sun in tents became surprisingly warm, they probably never attained the maximum temperature of those in

TABLE X

METEOROLOGICAL FACTORS INSIDE AND OUTSIDE TENTS ON TWO DIFFERENT DAYS.

Date	Location	Clock time	Air temperature in shade	Relative humidity	Solar radiation*
			°F	%	gm. cal./cm.²/min.
July 3, 1942	Inside tent	12:52 P.M.	....	....	0.798
	Outside tent	12:51 P.M.	89	43.5	1.330
	Inside tent	2:18 P.M.	....	....	0.751
	Outside tent	2:19 P.M.	90	42.0	1.285
July 6, 1942	Inside tent				
	Top of tree	11:08 A.M.	102	20.0	1.061
	Middle of tree	11:10 A.M.	102	19.0	1.021
	Bottom of tree	11:12 A.M.	101	19.5	1.000
	Outside tent	11:14 A.M.	100	19.0	....
	Inside tent				
	Top of tree	12:05 P.M.	108	18.0	0.837
	Middle of tree	12:11 P.M.	107	17.0	....
	Bottom of tree	12:12 P.M.	107	16.5	....
	Outside tent	11:57 A.M.	106	16.0	1.368

*Eppley thermopile measurements.

the open (table IX), total solar radiation in the tents being reduced between 38.8 and 41.5% (table X), and visible solar radiation being reduced between 26 and 27%. The severity of sulphur burn of fruit inside tents appears to have been reduced because few fruits attained temperatures high enough to cause much sulphur burn. No sunburn was found on fruit in the tents.

These results make it possible to estimate the effect of sulphur in lowering the threshold temperature at which injury occurs. If peel of nondusted fruit in direct sun inside tents reaches a temperature of 106° F without burning, and peel of nondusted fruit in the open shows a minute degree (0.78%) of sunburn at 113.1° F, while sulphur-dusted fruit in tents shows a fair degree of burn (3.58%), then the sulphur dust has lowered the threshold of injury of lemon peel from 113 to 106° F, or 7°. Such calculated values must be considered very rough approximations, however, for the fol-

lowing reasons: (a) Peel cells of citrus fruit (Valencia oranges) have been found by LEVITT and NELSON (**20**) to show considerable variability in the length of time they can withstand high temperatures; (b) It has been shown by TURRELL (**41**) and by TURRELL and CHERVENAK (**42, 43**) that the protein in lemon peel is involved in sulphur injury; and (c) The rate at which a

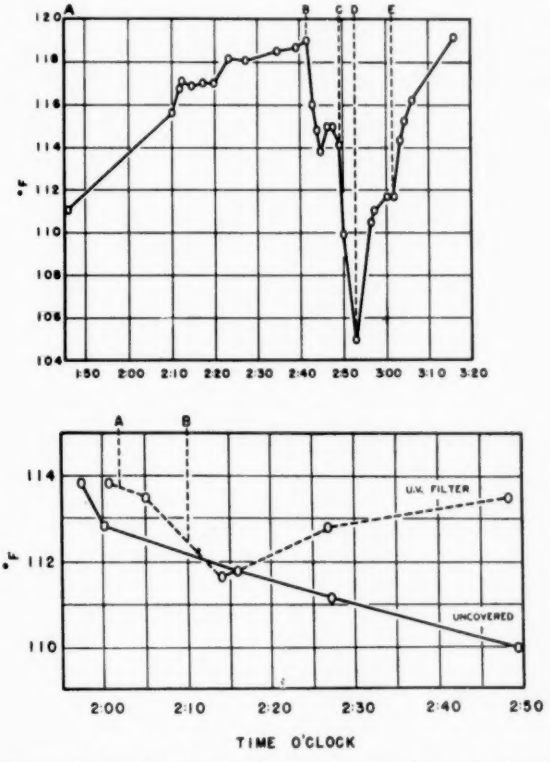

FIG. 5. Above: Peel-surface temperatures of a green lemon in direct sun July 3, 1942: fruit covered with ultraviolet filter immediately after first temperature reading (A); filter removed (B); opaque metal shield interposed between sun and fruit (C); shield removed (D); fruit again covered with ultraviolet filter (E). Below: Peel-surface temperatures of green lemons in direct sun July 12, 1942. Broken-line curve is fruit covered with ultraviolet filter after first temperature reading (A); continuous-line curve is fruit not covered with ultraviolet filter.

protein is heated is known to affect the temperature at which it is denatured (**29**). The sulphur burn noted above was caused by a temperature maintained at 111.7 to 113.8° F for one hour, but the rate of heating before these temperatures were reached is unknown.

EFFECT OF ULTRAVIOLET FILTERS.—On July 3, 1942, peel of an uncovered green lemon receiving solar radiation at the rate of 1.330 to 1.285 gm.

cal./cm.^{2}/min. had a surface temperature of 111.0° F (fig. 5, above). An ultraviolet filter was placed over the fruit, and within 55 minutes the peel surface reached a temperature of 119.0° F. The filter was removed, and the peel surface began to cool. A metal shield was then placed between the fruit and the sun, and with the solar radiant energy thus cut out the peel continued to cool. The metal shield was removed, and the temperature of the peel surface began to rise, leveling off at 111.7 to 111.9° F. The ultraviolet filter was replaced, and the temperature rose again to 119.2° F.

For comparison, peel-surface temperatures nine days later (on July 12), of an uncovered fruit and of a fruit covered with an ultraviolet filter, are shown in figure 5 (below). At 1:58 P.M. the peel of the uncovered fruit, which was receiving solar radiation at the rate of 1.358 gm. cal./cm.^{2}/min., had a surface temperature of 113.8° F, and the temperature continued to drop until the cessation of measurements. A similar lemon having a peel temperature of 113.8° F at 2:01 P.M. was covered with an ultraviolet filter at 2:04 P.M., and a surface temperature of 113.6° F was recorded for this fruit at 2:05 P.M. At 2:10 P.M. there arose a gust of wind having a measured velocity of 379 ft./min., and by 2:14 P.M. the peel temperature had dropped to 111.8° F. By 2:50 P.M. the wind velocity had dropped to an average of 326 ft./min., and the peel temperature had risen to 113.4° F.

As shown in table IX, on July 3 peel temperatures of filter-covered fruit in direct sun reached 119.2° F, whereas on a warmer day (July 6) the temperatures of similarly covered lemons reached only 113.8° F. Fruits at these temperatures reradiate at wave lengths of approximately 8.7 μ to 9.8 μ (**41**), and such radiation was acted on differentially by two factors which appear to be responsible for the high peel temperatures on July 3. The air-vapor density on July 3 was high (6.031 grains/cu. ft. of aqueous vapor), while on July 6 it was less than half as high (2.654 grains/cu. ft). The air velocity being low on July 3, water vapor from fruit transpiration was trapped between the filter and the fruit, thus increasing the already high air-vapor density. Water vapor absorbed infrared radiation (**8**) and maintained a layer of warm air around the fruit by absorbing infrared terrestrial and solar radiation (fig. 6 A and B), the latter being a little higher on the later date. Reradiation to fruit from the water vapor and from the ultraviolet filters, which also absorbed solar and terrestrial radiation, prevented dissipation of solar radiation received by the fruit (**41**). (The absorption region of the ultraviolet filter for terrestrial radiation of 7.0 to 11.0 μ in wave length is shown in fig. 4 B.) High fruit-peel temperatures thus developed through a double heat-trapping action of water vapor and ultraviolet filter.

On July 6, only the heat-trapping effect of the ultraviolet filter was important. The lower vapor density of the air on this day, and the gusty and higher wind velocity, prevented water vapor from accumulating under the filter and maintained a cooler filter by both contact conduction and radiation to the sky. Thus cooler fruit-peel temperatures were evident on this date. These points are brought out by comparison in figure 5 (above and below).

On the basis of the preceding temperature studies, it seems probable that during the periods of hot weather beginning on May 19 and July 4, the significant difference between injury to nondusted fruit covered with ultraviolet filters and to uncovered, nondusted controls was caused by the heat-trapping effect of the ultraviolet filter and of the water vapor trapped under it which resulted in high fruit-peel temperatures. Low air velocities generally prevailed during the morning periods (table II) and would have favored the trapping of water vapor transpired by fruit under the ultraviolet filters. Air-vapor density outside the filters was too low, however, to have had any

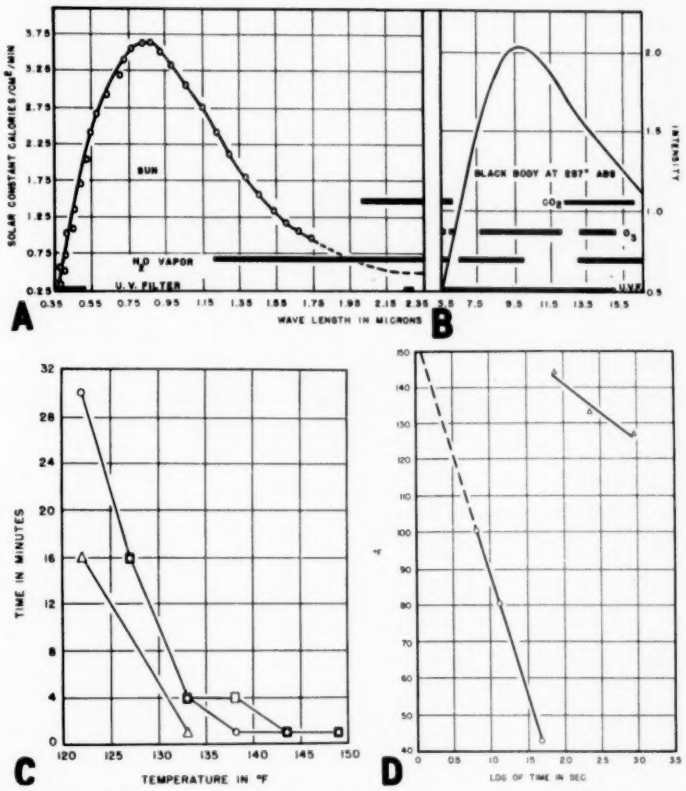

FIG. 6. A. Curve for envelope of solar energy (solar constant), from ABBOTT *et al.* (1). B. Curve for black-body radiation at 287° C, showing absorption range of atmospheric carbon dioxide, ozone, and water vapor, and of ultraviolet filter in visible and infrared regions. C. Time (in minutes) required to kill mature cortical cells of peel of Valencia orange (regular bloom, 18 months from set), at different temperatures, based on data of LEVITT and NELSON (20). Squares curve, 100% kill, July trial; circles curve, 100% kill, June trial; triangles curve, 50% kill, June trial. D. Time required for 100% kill of plant cells at different temperatures (°F). Triangles curve, albedo (cortical) cells of peel of non-sulphur-dusted Valencia orange (regular bloom) from LEVITT and NELSON (20); circles curve, Elodea cells treated with 0.8 *N* H_2SO_4, from CRAFTS (12).

significant influence in raising fruit temperatures by interfering with radiation to the sky.

The lack of significance between the percentage injury of sulphur-dusted fruit covered with the ultraviolet filters and sulphur-dusted uncovered fruit seems to be due to the fact that the critical temperature for sulphur burn was attained by all uncovered sulphur-dusted fruit exposed to direct solar radiation, so that the heat-trapping effect of the filters did not increase the incidence of sulphur burn. The burning under the ultraviolet filter indicates that ultraviolet radiation was not a factor, or was a very minor factor, in sulphur burn.

Effect of tree shape

Certain factors were not under good statistical control in the present experiments. One of the most important of these appeared to be the effect of tree shape on exposure of fruit to the sun. It was noted, for instance, that

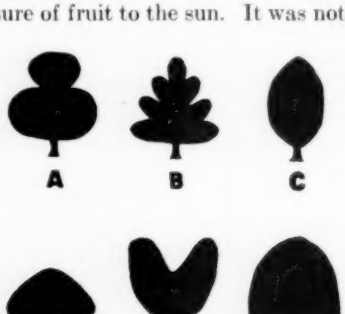

Fig. 7. Lemon-tree silhouettes: A. Shelved, B. Dispersed, C. Acuminate, D. Mushroom-shaped, E. Funnel-shaped, and F. Normal.

a tree acuminate in shape (fig. 7 C) had the smallest percentage of burned fruit, and that the compact, normal-shaped tree (fig. 7 F) also had a small percentage of sunburn or sulphur burn. Trees of irregular shape, on the other hand (fig. 7 A, B, D, E), which expose large numbers of fruit to the direct rays of the sun, had high percentages of sunburn and sulphur burn. Such factors as these play a very important role in influencing sunburn and sulphur burn, and influenced the statistical control of the experiment.

Discussion

The results presented show (a) that reduction of intensity of total solar radiation by the use of cheesecloth tents reduced sunburn and sulphur burn of lemon fruits, (b) that solar ultraviolet radiation is not requisite to sunburn or sulphur burn, (c) that sulphur burn is distinct from sunburn, and (d) that lime (with zinc sulphate) did not safen sulphur but caused a higher percentage of injury than sulphur dust. The last observation is in agreement with the observations of McGregor (**28**), who found that oranges were

more susceptible to injury by lime-sulphur than by sulphur dust. The small amount of leaf burn in the sulphur treatments, as compared with the fruit burn, is believed to be explainable on the basis of the greater buffer capacity of leaves (**47**), their lower temperature during solar irradiation, and consequently, their smaller absorption of sulphur vapor. Since it was shown that the amount of sulphur vapor produced by elemental sulphur increased logarithmically with linear increases in temperature (**40**), the role of the temperature of the plant part in making sulphur vapor available for absorption must be an important factor in sulphur burn.

A question of considerable importance is how long an exposure to direct solar radiation of a given intensity is required to injure non-sulphur-treated fruit. This, of course, depends upon a number of factors, of which the primary ones are size of fruit, fruit temperature at the beginning of irradiation, air temperature, air-vapor density, and rate of air flow. TURRELL (**46**) has shown the effect of fruit size on the rate of thermal increase. He reports that the surface temperature of the peel of a small lemon (volume, 31.5 cm.^{3}), at a starting temperature of 52° F, will attain a temperature 1 or 2° lower than that of the surrounding medium at 113° F in approximately seven minutes, whereas the peel surface of a large lemon (volume, 97.2 cm.^{3}), at a starting temperature of 46° F, will not attain a temperature of 113° F, under the same conditions, in less than approximately 60 minutes.

Curves published by TURRELL (**41**) show that a fruit having an initial temperature of 35° F, when immersed in a water bath at 118° F, attains a peel-surface temperature of 80° F in 240 seconds, and that a similar-sized fruit having an initial temperature of 95° F attains a peel-surface temperature of 107° F in the same time. LEVITT and NELSON (**20**) have shown that 25 to 50% of the albedo (cortical) cells of Valencia orange peel are killed by temperatures of 122° F in 16 minutes. Curves showing time and temperature required for 100% kill of mature cortical cells of Valencia orange peel, based on data of LEVITT and NELSON (**20**), are presented in figure 6 C.

The highest peel temperature recorded by TURRELL *et al.* (**44**) for fruit on the tree was 127.7° F, when the air temperature was 104.7° F. At this peel temperature, reference to figure 6 C shows that all the cortical cells (albedo cells between oil glands) would be killed by heat alone in 13 minutes. In four minutes 25% kill occurred, according to LEVITT and NELSON (**20**). By extrapolation of the upper curve shown in figure 6 D (**20**), downward to 119° F, the highest fruit-peel temperature recorded in the present experiment, a 100% kill of all the cortical cells of non-sulphur-dusted fruit could be expected in approximately 42 minutes. Since a peel temperature of 141° F might occur under rare conditions (**44**), it is interesting to note that all the cortical cells of non-sulphur-dusted fruit would be killed in approximately one minute after this temperature is reached (fig. 6 C and D).

A correlated question of considerable importance is how long an exposure to direct solar radiation is required to injure a sulphur-dusted fruit. No data are available on this point, so far as the authors are aware. However, a prognosis may be given if it is recalled that citrus fruits treated with

elemental sulphur dust, H_2S gas (formed by lime-sulphur spray, **2**), or SO_2 gas, and incubated, produce small amounts of H_2SO_4 in the peel (**41, 42, 43**). A restatement of the above question to include fruit treated with H_2SO_4 is therefore valid, and information on the effect of this acid is available.

CRAFTS (**12**), working with weed killers, showed that, for any given concentration of H_2SO_4, an increase in the temperature at which it was applied shortened the time necessary to kill the plant. His data for the killing time of Elodea leaves by 0.8 *N* H_2SO_4 are replotted in figure 6 D (lower curve) and extrapolated to temperatures which citrus fruit peel attains in direct sun on the tree. Also plotted in figure 6 D are LEVITT and NELSON'S data (**20**) for the minimum periods required for 100% kill of untreated albedo cells of Valencia orange peel at different temperatures. Comparison of these two curves shows that even such small amounts of sulphuric acid as are formed in lemon peel from sulphur dust or lime-sulphur spray would significantly lower the temperature and reduce the time in which injury occurs. The temperature and the time required to bring about sulphur injury to fruit on the tree lie between those shown by these two curves.

Sunburn and sulphur burn of fruit during the hot period of May 19 to 20, 1942, appear to have been caused by warm air (104° to 108° F) drifting in during the morning from the Mojave Desert north of the San Bernardino mountains. These mountains extend east to west and usually effectively impede the drift of warm air from the desert into the Santa Ana Canyon to the south. The total sun radiation on May 19 and 20 was lower than that of the three previous days (table II), and the outer air-vapor density was too low to have interfered substantially with fruit radiation to the sky, assuming that no change in O_3 or CO_2 concentration took place in the air. The meteorological data for this period and the results on sulphur burn of fruit inside and outside the tents, together with the results obtained with the ultraviolet filters, suggest that sunburn and sulphur burn were caused by the high temperature the fruit peel attained in the sun. This temperature was the resultant of the ratio of the rate of absorption of solar radiation by the fruit to the rate of heat loss by the fruit.

The radiation received by the fruit came from the visible, ultraviolet, and infrared regions of the solar spectrum, either directly or indirectly. The sun radiation curve based on the solar constant, as determined for the outer atmosphere by ABBOT *et al.* (**1**), is shown in figure 6 A. ELSASSER (**13**) states that, in practice, absorption of solar radiation by atmospheric gases is almost exclusively by water vapor. We have shown the solar constant because the absorption of solar radiation by the water vapor of the atmosphere is a variable predictable by the equation $A(u) = 0.172\ u^{0.303}$, for the range of 0.5 cm. to 8 cm. of precipitable water, where A is the solar energy absorbed in calories/cm.^{2}/min. and u is the column of precipitable water in grams/cm.^{2}. A curve somewhat similar to that of the solar constant, with an energy peak at 5000 Å, is given for the atmosphere at the earth's surface by BIRGE and JUDAY (**5**), but because of variations in atmospheric absorption it does not hold except under specially defined conditions.

Additional radiation in the infrared region was received by the fruit from the soil surface, tree foliage, and air. Of these, the most important contribution probably was made by the air. The CO_2 in the air absorbs solar radiation in the infrared region from 2 to 6 μ (**34**), and from about 12 to 16 μ (**33**), and is shown as a band in figure 6. It reradiates between 2 and 5.5 μ with a sharp maximum at 4 μ, and to a lesser degree between 13 and 15.5 μ, with a peak at about 14.5 μ. Total nonspectroscopic CO_2 emissivity is approximately 2% of black-body emissivity for 10 meters of atmospheric air, and 22% for 10,000 meters of atmospheric air (**13**), and thus probably contributes little to fruit temperature.

Ozone absorbs terrestrial radiation strongly between 4.3 and 5.5 μ (**4**), and at 5.9, 7.6, and 8.4 to 12.2 μ, the peak at 9.8 μ having the strongest (93%) absorption (**19**). There is also a fairly strong absorption band (65%) between 13 and 15 μ for an ozone column of 30-cm. length and 160-mm. pressure. Since these figures are for about 10 times the column normally present in the atmosphere, the actual atmospheric absorption due to this ozone band would be about 23% and therefore of minor importance in the radiative heat balance of the atmosphere (**13**), and likewise would contribute little to increasing fruit temperature.

The water vapor in the air absorbs infrared (terrestrial radiation at about 287° absolute, fig. 6 B) in bands of varying proportions, from 1.2 to 5.5 μ and from 6.4 to 8.3 μ (**15**); it absorbs fairly well to about 10 μ; from 10 μ to about 13 μ it is quite transparent to infrared, and then absorption is increasingly strong up to wave lengths of 100 μ (**13**). Strong absorption bands occur between 45 and 120 μ, especially at 50, 66, and 79 μ, while high transparency occurs at 47, 54, 62, 75, 91, and 115 μ (**33**). However, the absorption bands of water vapor vary somewhat with temperature (**13**). The emissivity of water vapor (nonspectroscopic) at 20° C ranges about 8% of black-body emissivity for 0.001 gm./cm.^{2} of precipitable water in the atmosphere to 54% with 0.3 gm./cm.^{2} At 15° C and 0.0001 gm./cm.^{2} precipitable water, the emissivity is 1.3% of black-body radiation, and at 0.0025 gm./cm.^{2} emissivity, it is approximately 15% (**13**). The absorption and emission of nitrogen and oxygen in the atmosphere appear to be very small, and of water vapor, very large. The effect of water vapor on terrestrial radiation was shown by Brooks (**7**) to be approximately 0.113 gm. cal./cm.^{2}/min. to a clear sky and 0.0136 gm. cal./cm.^{2}/min. to a cloudy sky (our calculations).

From the above discussion, it may be seen that fruit temperature may be reduced by radiation release from the fruit (a) to water vapor, CO_2, etc. (**9**), to the air and its constituents; (b) to soil, trees, and sky; and (c) by mass movement of the air (contact conduction) if these are cooler than the fruit. For leaves exposed to the sky, radiation to the sky may be the most important factor in reducing their temperature, since exchange of heat by radiation is governed by a law (Wien's) in $T_1^4 - T_2^4$. Both T_1 and T_2 would be important in applying this equation where the vapor density of the air is low and there is approximately a 300° difference between leaf temperature

and sky temperature. However, if there is considerable warm water vapor present in the atmosphere, the temperature difference between plant part and water vapor is small, and radiation proceeds according to the simple law $T_1 - T_2$ (**51**). Contact conduction was probably the most important for fruits in this study, as they exposed less of their total surface to the sky than leaves. The air was hot and dry on May 19 and 20; and since solar radiation was lower on these two days than previously, the hot air must have been effective in bringing about sunburn and sulphur burn on May 19 and 20 by retarding release of absorbed solar radiation as terrestrial radiation (heat) by the fruit. Fruit burn, however, on July 4 to 10 was effected not only by warm, dry air drifting in from the desert, as on May 19 and 20, but also by increased intensity of solar radiation (table II).

Physiological factors, as well as physical factors, influence the variability in injury of citrus fruit of various sizes and colors. There is a correlation between fruit size, color of peel, maturity of fruit, physiological activity, and chemical composition. Maturity was shown by LEVITT and NELSON (**20**) to affect injury by heat. Their recorded data indicate a greater susceptibility to high-temperature injury in peel cells of 18-month-old oranges (regular bloom) than in peel cells of 12-month-old oranges (June bloom).

MCGREGOR (**28**) likewise observed that maturity affected susceptibility to injury. He found that small green oranges were more susceptible to sunburn and sulphur burn than fruit of other degrees of maturity. This agrees with an *a priori* assumption based on their small size and their greater absorption of visible radiation. Lack of statistically significant differences between sulphur burn of green fruit and yellow fruit, and of large fruit and small fruit, in the present experiments, are thought to be due to the small number of trees, and to the small number of yellow fruits, all of which were picked prior to the initiation of the experiment.

Catalase activity is lower in the peel of yellow lemons than in that of green lemons (**45**). Elemental sulphur dust has been shown to inactivate catalase (**45**). Since it is generally known that the catalytic rate of an enzyme is a function of concentration, it is possible that this enzyme because of its low concentration in yellow fruit would be more quickly inactivated by sulphur dust in the peel of yellow fruit than by that in the peel of green fruit.

There is very little data in the literature on the inorganic composition of the peel of developing citrus fruit. Calculations made on lemons during maturation (**3**), however, show a slight increase in the ash content in peel of older fruit, accompanied by an increase in calcium and magnesium. Potassium and sodium percentages remained the same or decreased slightly. COPEMAN (**10**) found that an increase in the ash of the juice of ripening Washington Navel oranges was accompanied by an increase in potassium. HAAS and KLOTZ (**17**) found increases in percentages of ash, sodium, and potassium in the juice of mature Valencia oranges in a one-month period, and in the peel of Eureka lemons over a seven-month period. The increase

in inorganic cations during fruit development should decrease the susceptibility of mature fruit to sulphur burn.

It has been shown by TURRELL (**41**) and by TURRELL and CHERVENAK (**43**) that there is an increase in acid concentration in the peel of sulphur-dusted fruit incubated at high temperatures. The protective device against sulphur burn in the peel appears to be its buffer capacity. Though low in the peel, buffer capacity appears to reside primarily in the organic acids and their salts. An increase in the organic acid salts during fruit development would increase the buffer capacity of the peel and increase the resistance to sulphur burn in mature fruit peel. High catalase activity in the peel of small green fruit therefore seems to be its only protective device. Available biochemical and physical data otherwise indicate the greater susceptibility of these fruits to sulphur burn, as observed by MCGREGOR (**28**).

Summary

A field experiment was designed to show the effect of reduction in intensity of total solar radiation, and of reduction in ultraviolet intensity in solar radiation, on sunburn and sulphur burn of lemon fruit.

Lemon trees of a mixed population were dusted with elemental sulphur; some of these trees were covered by cheesecloth tents; some were left uncovered, and fruits exposed to solar radiation on some of the uncovered trees were covered by ultraviolet filters. Trees of a second lot, not dusted with sulphur, were covered with cheesecloth tents, or exposed fruits were covered with ultraviolet filters. Trees of a third lot were sprayed with lime-sulphur (plus zinc sulphate), but no coverings of any kind were applied.

Fruits exposed to direct solar radiation where there was no reduction of normal intensity were generally injured, although foliage showed very little injury whether treated with sulphur or not. Lime-sulphur spray caused the greatest percentage of sulphur burn. Elemental sulphur dust increased the percentage of burn from sun radiation. Reduction of total solar radiation reduced sunburn and sulphur burn, but elimination of the ultraviolet radiation in the solar spectrum did not reduce either sunburn or sulphur burn. The ultraviolet filters behaved as heat traps. Higher percentages of sunburn and sulphur burn were found on south and top sides of trees than on other sides. No significant difference was found between susceptibility of large and small fruit, or of yellow and green fruit, to sunburn or sulphur burn. Likewise, no significant differences in burning occurred in the May and July hot periods.

Sulphur treatment appeared to lower the temperature at which solar radiation injures lemons. Injurious fruit-peel temperatures are suggested as due to an unfavorable ratio of energy received to energy lost by fruit. In this experiment warm air was shown to be the primary factor interfering with heat dissipation by fruit. Effects of other meteorological factors are discussed.

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STUDIES ON THE CHEMISTRY OF THE LIVING BARK OF THE BLACK LOCUST TREE IN RELATION TO FROST HARDINESS. IV. EFFECTS OF RINGING ON TRANSLOCATION, PROTEIN SYNTHESIS AND THE DEVELOPMENT OF HARDINESS[1]

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Seasonal changes which occur in the water-insoluble protein nitrogen, the water-soluble protein nitrogen, the water-soluble non-protein nitrogen, and the reducing and non-reducing sugar contents of the living bark tissue of normal black locust trees and the relationships that these changes bear to seasonal variations in the frost hardiness of this tissue have been reported earlier (**2, 22**). These studies were initial steps in an analytical approach to the problem of the biochemistry of frost hardiness. The objective of this approach is the identification of those changes in biochemical composition of the cells which occur in association with their changes in hardiness. Such information, while not necessarily supplying a basis for explaining the physiological mechanism involved in hardiness, constitutes a necessary advance in the attempt to elucidate this mechanism.

Among the seasonal changes in the chemical composition of the tissues which were followed, those which occurred in the water-soluble proteins showed the best correlation with frost hardiness changes. This relationship could have been fortuitous rather than causal, *i.e.*, it could have arisen from a simultaneous but independent normal seasonal variation in the protein content of the cells and in their hardiness. Included in these earlier studies were results of hardiness tests and chemical analyses performed on the bark of the stumps of trees from which the crown and upper trunk had been removed in winter. Dehardening of the bark of the stumps in the spring was observed to be abnormally slow and the seasonal decrease in protein which is observed in normal trees was also delayed in proportion to the observed retention of hardiness. This apparently resulted from the elimination of the upward translocation of bark cell constituents from the trunk to the crown which normally occurs in the spring in response to the demands made by the new flush of growth. Even under such unseasonal conditions, the association of soluble protein content with hardiness was observed. Contrasted to this,

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the soluble carbohydrate content of the stump tissue dropped to values characteristic of the dehardened tissue of normal bark at this time of year. These observations, combined with the evidence obtained from the seasonal studies on normal trees, emphasized the possibility that the concentration of water-soluble protein in the cells was truly a causal factor in their frost hardiness and made it desirable that further evidence be obtained through studies made under other unusual circumstances and conditions than those which were provided in the stump experiments.

The effect of hindered translocation in producing the unseasonal sequence of events in the bark of the stump suggested the form which further experiments should take in testing this relationship. Essentially the experiments which are reported here consist of a study of the relationship of protein concentration to the hardiness developed in early winter in the intercepted bark sections of trees multiply ringed at various times during the summer and early fall. Included in this study are those changes which occurred at the same time in the soluble sugars and starch under these conditions. Experiments were conducted over a period of two seasons extending from July 1949 to March 1951. Those begun in the summer of 1950 were more extensive than those begun in 1949 and in them the attempt was made to answer some of the questions which were derived from the preliminary experiments in 1949.

Materials and methods

Experimental tissue for these studies was prepared during the summer by periodic and successive ringing of the bark of the trunks of black locust trees, starting near the base of the trunk and progressing upward as later rings were cut. Trees selected for the experiments were about 15 years old and 20 to 30 feet high. Each of the rings was made as follows. At a selected point along the trunk a ring of whole bark two or three inches in width was cut and pried away from the wood without injury to the underlying xylem. The gap so formed between the bark above and below the ring was of sufficient width to insure that reunion by extension of meristematic growth from the cambium could not take place. The exposed wood surface of the ring was scraped to remove any remaining traces of connecting strands of phloem or cambial tissue. After a short drying period shellac was applied to the cut edges of the bark adjoining the wood as well as to the wood surface. The time sequence of ringing varied to some extent but one example will serve to illustrate the general procedure followed. Ringing was initiated on August 11 at which time two rings were cut, one at a distance of three feet from the ground and a second three feet above the first. Thus a section of the bark, while left in place on the trunk, was isolated so that translocation of those substances which normally would be conducted through the phloem to or from this section was stopped. Additional rings were made on August 19, August 24, September 2 and September 22, each at a distance on the trunk three feet above the preceding ring. In this manner a series of five sections of bark, each isolated by rings above and below, were left on the trunk. The basal part of the bark below the first ring remained in contact

through the phloem with the roots, and the part of the trunk above the last ring remained in like contact with the crown and leaves.

The effect of ringing a tree in this manner was that the contact with the roots through the phloem was severed for all parts of the tree above the first ring at the time when it was cut, August 11 in the example given. On the other hand, phloem continuity with the crown of any part of the bark above a given ring was not interrupted until such time as a ring was cut above that part. Thus the various sections, isolated by rings above and below, differed from one another only in the time interval during which contact with the leaves and the crown was maintained through the phloem, the later ringed sections having the longest period of contact. The crown section of the bark retained such contact with the leaves until normal abscission.

The primary purpose of these ringing experiments was to produce such unseasonal variations in the chemical composition and hardiness of the bark of the ringed sections as to permit a further examination of the relationship of the soluble protein content of the bark to its frost hardiness. The design of the experiments was such, also, as to yield information on the well-known phenomenon of autumnal migration of carbohydrates and nitrogenous substances and on those physiological and chemical changes which can occur in the bark independently of phloem-transported nutrients from the leaves and roots.

While some defoliation in summer and a small amount of premature yellowing and leaf abscission sometimes resulted from ringing, moisture analyses of the ringed sections and the turgidity of the leaves of the ringed trees indicated that conduction of water through the xylem was not appreciably impaired by ringing of the bark and was sufficient for the transpirational demands of the crown of the tree. In the black locust tree the current year's deposit of xylem retains its capacity to transport water from roots to crown. Older layers of wood lose this ability, their passages becoming plugged with tyloses (**5**). Bark ringing in the manner described, if performed before July 1, led to a water starvation of the crown, with severe leaf abscission shortly after the ringing. Performed after the middle of July, however, such ringing led to little water starvation, indicating that the new wood was then extensive enough to carry the water required by the crown after that period.

The doubly ringed sections of the bark, while isolated insofar as transport through the phloem was concerned, remained in contact with the xylem system transporting water from roots to crown. The possibility existed, therefore, that some exchange of small molecular substances might occur by diffusion between the xylem region and the isolated bark sections. Any such substances reaching the cells of these isolated sections, however, would be derived, perhaps exclusively, from reserve supplies in the roots and such supplies appear to be relatively of little importance in determining the composition of the bark cells compared to those which normally move down through the phloem from the leaves at this period of the year.

The relative capacities of the experimental tissues to resist frost injury were estimated by the use of dehydration tests (**23**). While the methods followed in preparing the experimental tissue and in estimating the relative hardiness of the bark cells were similar in the studies conducted during both years, other experimental procedures as well as the objectives of the experiments varied in the two cases.

1949 Experiments

Two trees were used in these experiments. The segments of the bark on each tree were isolated by ringing on the following dates: August 11, August 18, September 3, and September 23. In November, at which time most of the hardening and the chemical changes associated with hardening are complete in normal trees, hardiness tests (plasmolysis) were performed on the tissues of the various segments of the bark, including the sections still in phloem continuity with the roots and the crown. The trunks were then cut into log sections at the points of ringing, and sections of the crown and of the stump were taken. From each segment, samples were obtained for analysis by removing the dead outer bark from a section of the log (at a distance of six to eight inches from the ring) and grinding off the living bark with a wire brush wheel. The logs, still retaining most of the bark undisturbed, were then stored out of doors in order to determine their survival after winter exposure as a check on the plasmolysis hardiness tests. In March, survival was estimated and samples were again taken for analysis from those sections that had survived the winter.

The samples collected in November and March were stored in glass jars at – 16° C prior to analysis. Moisture was determined by drying a portion of the sample in a vacuum oven at 105° C for six hours. Nitrogen fractionation was carried out on a portion of the sample (equivalent to 4 to 5 grams dry weight) by thorough extraction with 150 cc. of distilled water, filtering, reextraction of the residue with 50 cc. of distilled water, filtering and washing the residue. Trichloroacetic acid was added to the total filtrate to make a 10% solution, the precipitated protein was collected on a filter paper and washed with a 10% trichloroacetic acid solution. Kjeldahl nitrogen was determined on the residue from the water extraction (insoluble protein N), on the trichloroacetic acid precipitate (water-soluble protein N) and on the filtrate from the trichloroacetic acid precipitate (non-protein N). Sugars were determined by the method described by Hassid (**6**) which yielded lower values for total sugars than those reported earlier for similar tissues (**22**) due to the circumstance that in the earlier experiments the method of extraction, employing acid, was accompanied by some hydrolysis of the non-sugar carbohydrates present.

1950 Experiments

The objectives in 1950 were to obtain more extensive data on the effects of ringing in summer on the final hardiness and chemical composition of the bark in late autumn and, in addition, to determine the total changes in

hardiness and composition of the bark which occurred between the time of isolation by ringing and the same time in the autumn.

A total of 12 trees, six from each of two groves located some 20 miles apart, were used. With some of these trees the ringing and isolation of bark sections was initiated as early as July 7. With most, however, the period during which the ringing was done was approximately the same as in the experiments of 1949. Samples of the living bark corresponding to the segment being isolated were taken for analysis on the date of isolation. The analyses and tests of these samples for hardiness were compared with those of samples taken later in November from the corresponding isolated segment and showed to what extent composition and hardiness changes occurred in these segments subsequent to isolation and up to the time when the bark of normal trees had essentially completed hardiness development. Analyses for moisture, for the nitrogen distribution between the insoluble protein, soluble protein and non-protein fractions, and for soluble sugars were made as in 1949. In addition, determinations of starch content were made.

The requirement that a considerable number of samples corresponding in composition to each newly ringed section be collected at the time of isolation made it necessary to adopt a different sampling procedure. Since these sections were to remain on the tree throughout the summer and early autumn, it was necessary to avoid excessive mutilation of the bark at the time of ringing in order to minimize the risk of impairing the water supply through injury to the current year's xylem and to minimize the risk of injury to the tissues through access which could be provided to decay organisms. These difficulties were largely circumvented by the use of very small samples and by a different technique for removal of these samples. Previously, sampling had been done by cutting whole lengths of logs from the trees and grinding samples from the exposed living bark with a wire brush wheel. Because of the intensive disintegration of the bark in this case, extractions for analyses could be done without further maceration of the tissues. In the present experiments small samples of whole bark were taken instead directly from the tree and a different extraction technique was required. Previous tests had indicated the extent to which these samples could be reduced in size and still yield analyses and hardiness values which were representative of the adjacent bark tissues. In every case, however, duplicate analyses and hardiness tests were made on tissues from adjacent areas as a further check against the occurrence of abnormal variations. In order to preserve intact as much of the bark tissues as possible at the time of ringing, the samples for analyses and tests of hardiness were taken, previous to ringing, from that portion of the bark which was designated for removal as the ring. Because of the small size of the samples, sufficient bark tissue was available for this purpose without enlarging the ring.

At the time of ringing the dead bark at the location chosen for the ring was scraped away to expose the living bark, and a series of approximately equal samples of the living bark (about 0.5 gram fresh weight and about 3 to 4 square centimeters in area) were carefully removed from the wood

and transferred to sampling bottles. The ring was then completed by discarding the remaining bark assigned to the ring. But for this variation in sampling, multiple ringing was carried out in a manner identical with that previously described. Hardiness tests were made on these tissue samples within a few hours after collection. The remaining samples were stored at $-16°$ C until analyzed.

In November, when the final chemical composition and hardiness of each of these sections was to be determined the trees were felled and cut into logs at their points of ringing. Samples were collected from each section at a point about 12 inches below the location of the upper ring, and from that part of the trunk above the last ring (crown) and below the first ring (stump). Hardiness tests and analyses on these samples were made as with the earlier collected tissues.

The logs were stored outdoors until March when an estimate of the survival after exposure to winter conditions was made as a check on the hardiness tests made in November. Because severe freezing conditions were not experienced during that winter, a proper differentiation of the hardiness in the various sections was not obtained although such a differentiation was predicted by the November tests. The logs were therefore stored at a temperature a few degrees below the freezing point from March until June when a freezing unit became available in which much lower temperatures than those attained outdoors could be produced. A better differentiation of the hardiness of the various sections was then obtained which confirmed the hardiness predicted by the tests made in November. Only the results of the laboratory hardiness tests obtained in November (both the plasmolysis and desiccation tests were employed) are reported in this paper.

Some defoliation was caused by the ringing but this was not considerable except in one tree (no. 1–B) on which the initial rings were made on July 7. With the other trees, retention of turgidity in the leaves and of normal moisture content in the bark throughout the summer indicated that essential water-conducting tissues had remained intact to the extent required to supply the water demands of the living tree.

Because the samples taken were whole pieces of bark, maceration of the tissue was required prior to extraction in some of the analytical determinations. The methods adopted varied with the nature of the analyses performed. Moisture was determined, as before, by drying a portion of a sample in a vacuum oven at $105°$ C for six hours. No pretreatment of the sample was required for this determination. In the determination of soluble sugars and starch the samples were finely sliced with scissors previous to extraction in hot 80% alcohol, although complete extraction of the sugars could have been obtained on the intact samples. The total soluble sugars, including reducing and non-reducing sugars, were determined by Hassid's method (**6**) subsequent to treatment of the extract, after removal of the alcohol, with invertase. After extraction of the soluble sugars, the sliced samples were extracted with ether, dried, and ground thoroughly with sand

in a mortar. Starch was then extracted with perchloric acid and determined colorimetrically with I_2 by the method of NIELSEN (**16**).

Some departure from the previous methods used in extraction of the various nitrogen fractions was required. Denaturation of the water-soluble protein had to be avoided if a true estimate of its amount in the samples was to be obtained. For this reason extraction of the protein from fresh samples would have been desirable. Because the number of samples collected at any one time was large, extractions could not be made immediately. The samples were therefore rapidly frozen and stored in the frozen state until they were to be extracted. Electrophoretic analyses and quantitative determinations on the protein extractable from fresh and from frozen samples indicated that no denaturation or other changes were entailed by the freezing and storage of the samples. Before extraction, the samples were thawed, rapidly ground under water in an agate mortar, transferred to a centrifuge tube and exhaustively extracted and washed with water by decantation and centrifugation. The residue contained the water-insoluble protein. The water-soluble protein in the collected extracts and washings was precipitated by addition of trichloroacetic acid to 10% concentration and the precipitate was separated by centrifugation, decantation and washing with 10% trichloroacetic acid solution. The supernatant and washings from this precipitate contained the non-protein water-soluble nitrogen. The nitrogen content of each of these fractions was determined by a micro-Kjeldahl procedure.

Results

The extent to which variations in the chemical composition of bark tissue occur at different heights along the trunks of normal, unringed trees was first established. The nitrogen fractionation analyses for two such trees are shown in table I, the samples having been taken in duplicate at various heights along the trunks from base to crown. One tree was sampled in July, the other in January. These analyses indicate that the normal variation in the chemical composition of the living bark cells at various points along the trunk of a given tree at a given time is slight and well within the errors of the sampling and extraction procedures employed. Analyses for sugars and starch show similar uniformity (**24**). For the purposes of the present studies, it is evident that any variations exceeding approximately $\pm$ 10% that may be observed in the composition of the ringed sections of a given tree must arise as a consequence of the ringing process and not from normal variations along the trunk.

Variations from one tree to another at a given time of year may be considerably greater than those observed between the various parts of the trunk of a single tree. Table II lists nitrogen distribution analyses made on bark samples taken near the base of several different trees but all at the same time of year and under similar extraction procedures. These variations reflect, undoubtedly, differences in the nutritional state, general health and

TABLE I

NITROGEN CONTENT (DRY WEIGHT BASIS) OF BARK TISSUES OF NORMAL TREES AT VARIOUS LEVELS ALONG THE TRUNK.

Hardiness	Part of trunk sampled	Insoluble protein nitrogen	Soluble protein nitrogen	Non-protein nitrogen
		%	%	%
Low (July)	Near crown	1.08	0.58	1.12
		1.07	0.52	1.12
	Upper middle	1.14	0.54	1.07
		1.08	....	1.04
	Lower middle	1.04	0.61	0.93
		1.02	0.58	0.96
	Base	1.14	0.64	1.04
		1.10	0.57	1.04
	Average	1.08	0.59	1.04
High (January)	Near crown	1.12	1.12	0.75
		1.14	1.11	0.83
	Upper middle	1.15	1.15	0.80
		1.12	1.15	0.70
	Lower middle	1.15	1.14	0.69
		1.13	1.07	0.68
	Base	1.08	1.09	0.74
		1.09	1.09	0.67
	Average	1.12	1.11	0.73

vigor, and environmental conditions together with normal biological variability within the species population. It is apparent, therefore, that in comparing effects due to hindered translocation in the ringing experiments, more significance may be accorded observations made on an individual tree than those made on different trees even though similarly ringed on identical dates. The general trend of these effects would, however, be expected to be similar for all individuals.

TABLE II

NITROGEN CONTENT (DRY WEIGHT BASIS) OF BARK TISSUES OF DIFFERENT NORMAL TREES SAMPLED AT A GIVEN TIME OF YEAR.

Date of sampling	Insoluble protein nitrogen	Soluble protein nitrogen	Non-protein nitrogen
	%	%	%
July 15	1.12	0.60	1.04
July 15	1.10	0.48	0.80
July 15	1.20	0.45	0.90
July 15	1.02	0.40	0.65
January	1.28	0.90	1.23
January	1.06	0.97	1.02
January	1.18	0.90	1.17
January	0.98	1.10	0.75
January	1.08	1.09	0.70

1949 Experiments

Results obtained in the experiments conducted on two trees, periodically ringed in the summer of 1949, where the tissues of the various segments were tested for hardiness and analyzed in November and again in March 1950, are given in table III. Hardiness, as measured by the plasmolysis test in November, is in terms of the molarity of the most concentrated salt solution to which the tissues could be subjected and have 100% survival of the cells. Hardiness as measured in March was estimated in terms of per cent. survival of the cells after being subjected on the log, out of doors, to winter conditions. Analyses were not made on the tissue from the base of the tree in November and were not made in March on the tissues of those segments which were killed by winter conditions. Briefly, these studies showed the following: (a) Interception of phloem continuity of the bark with the crown and roots had no effect upon the insoluble protein content of the tissues as measured later in November and in March. (b) Ringing had no consistent effect, if any, on the non-protein nitrogen content of the cells at these times of analysis. (c) Ringing had a marked effect on the extent to which the cells of the various bark segments were able to accumulate both water-soluble proteins and soluble sugars, those segments earliest isolated from phloem continuity with crown or roots being most deficient in these abilities. (d) Plasmolysis tests for hardiness made on the tissues of the various segments in November indicated that their relative hardiness was proportional to their relative content of water-soluble protein at that time. (e) Some increase in soluble protein occurred between November and March in the sections ringed later and in the section which had remained in contact with the crown, but actual survival under winter conditions followed roughly their hardiness as indicated in November by the plasmolysis test. (f) The base section, which remained in contact with the roots through the phloem, showed, in marked contrast to the ringed section immediately above it, a high capacity to acquire hardiness and concurrently to accumulate water-soluble protein. Reserves, not present at the time the first ringing was done, were apparently mobilized from the roots to make this synthesis possible. (g) Soluble sugar contents of the tissues of the various segments showed, in November, a correlation with the estimated hardiness, but in March the sugar contents of many of the sections which were hardy and had survived winter conditions had dropped to values comparable with those of tissues which is November showed low hardiness and which were unable to survive the winter. This is in contrast to their soluble protein contents and serves to indicate that soluble sugars, if they contribute to hardiness at all, are secondary in this function to the contribution made by the water-soluble proteins.

1950 Experiments

The objectives of the studies in 1950 were (a) to seek confirmation of the preliminary results through use of a greater number of trees in the experiments and (b) to determine what changes occur in the chemical composition and hardiness of the ringed sections between the time at which the

TABLE III

ANALYTICAL AND HARDINESS DATA ON THE BARK TISSUES OF TREES MULTIPLY AND PERIODICALLY RINGED IN THE SUMMER OF 1949. ANALYSES ARE GIVEN AS PER CENT. OF DRY WEIGHT AND ARE AVERAGES OF DUPLICATE DETERMINATIONS.

Ringed tree	Time of ringing	Part of tree	Insoluble protein nitrogen		Soluble protein nitrogen		Non protein nitrogen		Soluble sugars		Hardiness	
			November	March	November	March	November	March	November	March	November molarity	March survival
			%	%	%	%	%	%	%	%		%
1	September 23	Crown	1.12	1.03	0.83	1.09	0.73	0.70	11.5	10.5	5.0	100
	September 3	4	1.16	1.08	0.77	0.83	0.94	0.79	11.0	9.0	5.0	100
	August 18	3	1.16	1.26	0.58	0.67	1.07	1.16	8.2	6.2	4.5	100
	August 11	2	1.19	....	0.50	....	1.25	....	6.8	....	3.5	25
	August 11	1	1.17	....	0.54	....	1.17	....	4.5	....	3.0	0
		Base	....	1.28	....	0.90	....	1.46	....	6.7	4.8	100
2	September 23	Crown	1.06	1.10	0.80	0.98	1.05	1.14	11.2	10.5	5.0	100
	September 3	4	1.10	1.12	0.75	0.88	1.11	1.07	10.5	8.5	5.0	100
	August 18	3	1.11	1.12	0.61	0.62	1.20	1.11	9.2	5.5	4.5	100
	August 11	2	1.13	....	0.47	....	1.10	....	5.6	....	3.0	35
	August 11	1	1.12	....	0.48	....	1.09	....	5.0	....	2.5	0
		Base	....	1.06	....	0.97	....	1.05	....	10.5	4.5	100

TABLE IV

ANALYTICAL AND HARDINESS DATA ON ONE TYPICAL RINGED TREE (NO. 4-R) IN THE 1950 EXPERIMENTS. ANALYTICAL DATA ARE PER CENT. OF DRY WEIGHT.

Date of ringing	Part of tree	Insoluble protein nitrogen		Soluble protein nitrogen		Non-protein nitrogen		Soluble sugars		Starch		Dry weight		Hardiness relative vapor pressure for 50% killing	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B
		%	%	%	%	%	%	%	%	%	%	%	%		
September 21	Crown	....	0.99	....	1.08	....	0.75	....	10.7	....	5.7	....	48.7	....	0.80
September 2	4	1.01	1.12	0.74	1.07	0.84	0.81	7.0	10.2	15.6	3.4	47.9	46.5	0.91	0.81
August 24	3	1.03	1.14	0.52	0.72	1.05	1.06	8.6	8.1	10.0	2.0	40.2	42.6	0.94	0.85
August 15	2	1.10	1.05	0.49	0.71	1.07	0.93	10.4	7.6	8.3	0.9	39.7	41.0	0.95	0.88
August 15	1	1.05	1.13	0.55	0.59	1.00	1.17	7.3	4.6	1.8	0.3	35.9	39.1	0.98	0.89
	Stump	1.05	1.16	0.55	0.90	1.00	1.18	7.3	7.5	1.8	0.3	35.9	42.7	0.98	0.83

Columns A and B under each heading refer respectively to values determined on the bark section at the time of isolation by ringing and on the same section after it had remained on the tree until mid-November.

section is isolated and the time in November when the summer cycle is complete. From these experiments information was obtained, also, regarding the importance of the autumnal migration of substances synthesized in the leaves to the development of hardiness and to the synthesis of water-soluble proteins in the bark cells.

While the studies were conducted on 12 trees in the experiments of 1950 and the results from all are taken into account in the conclusions drawn, results for only a few examples can be presented. In table IV are given the initial (at time of ringing) and final (mid-November) analyses for the nitrogen fractions, for soluble sugars, for starch, the dry weights of the tissues, and the hardiness (desiccation test) of the bark of the ringed sections of one tree (no. 4–R) which was typical and representative of the majority. In table V are shown the initial and final analyses of the bark segments of four other trees for soluble sugars, for starch and for soluble proteins together with the hardiness of the corresponding tissues. Results on trees no. 5–R and no. 3–B are representative, as are those on tree no. 4–R given in table IV, of typical findings on the 12 ringed trees used in the 1950 experiments. Results on trees no. 1–B and no. 3–R are given because they represent atypical examples. Tree no. 1–B, first ringed on July 7, suffered severe defoliation. Tree no. 3–R is atypical in that even its earliest ringed sections showed normal protein synthesis and hardiness development in November, which is in contrast to the findings on the early ringed sections of the typical trees. The data for tree no. 3–R showed a maximum of variation from that which is considered typical with regard to soluble protein synthesis subsequent to isolation of the sections. It is significant that, in this tree where the hardiness variations between the segments in November were also atypical, a definite positive relationship nevertheless exists between the increase in soluble protein content and the increase in hardiness of the tissue which occurred between the time of isolation of the segment and in November.

The existence and general nature of this correlation between hardiness and the water-soluble protein content of the bark tissues is illustrated by plotting, as in figure 1, the water-soluble protein nitrogen (per cent. of dry weight of the tissue) against the relative vapor pressure (in the desiccation test for hardiness) required to bring about 50% killing of the tissues at the various times and under the various conditions investigated in this study. The spot diagram is diffuse, as is to be expected in such a comparison where natural variations between trees and the errors of both the analysis and hardiness test are involved. Two points of special significance, however, are recognized from this treatment of the data. (a) Not all of the water-soluble protein of the tissue is effective in promoting hardiness. An amount of soluble protein equivalent to approximately 0.5% nitrogen contributes little or not at all to hardiness. This value for the non-effective soluble protein corresponds closely to the average value in early summer tissue where hardiness is minimal for the year. (b) The degree of hardiness increases strongly

TABLE V

ANALYTICAL AND HARDINESS DATA ON FOUR TREES IN THE 1950 EXPERIMENTS. ANALYTICAL DATA ARE PER CENT. OF DRY WEIGHT.

Ringed tree	Date of ringing	Part of tree	Hardiness relative vapor pressure for 50% killing		Water-soluble protein nitrogen		Soluble sugars		Starch	
			A	B	A	B	A	B	A	B
					%	%	%	%	%	%
5-R	September 9	Crown	0.89	0.77	0.75	1.03	6.2	12.4	16.0	5.2
	August 15	2	0.92	0.81	0.58	0.71	7.6	9.8	11.5	1.5
	August 15	1	0.96	0.88	0.54	0.59	5.9	4.6	1.5	0.4
		Stump	0.96	0.81	0.54	0.91	5.9	10.4	1.5	0.3
3-B	September 21	Crown	....	0.78	....	0.96	....	12.0	....	4.5
	August 29	3	0.95	0.78	....	0.95	....	10.5	....	1.2
	July 28	2	0.97	0.84	0.60	0.80	6.6	6.5	9.1	0.2
	July 28	1	0.98	0.88	0.58	0.74	5.5	4.7	1.1	0.1
		Stump	0.98	0.80	0.58	0.85	5.5	9.0	1.1	0.2
1-B	August 18	Crown	....	0.79	....	0.90	....	8.3	....	0.4
	July 27	3	0.97	0.81	0.60	0.82	6.8	6.0	12.5	0.3
	July 7	2	0.97	0.84	0.57	0.62	7.2	5.1	7.4	0.4
	July 7	1	0.96	0.91	0.49	0.52	7.9	1.2	0.6	0.3
		Stump	0.96	0.81	0.49	0.71	7.9	9.5	0.6	0.3
3-R	September 9	Crown	....	....	....	....	....	....	....	....
	September 2	4	....	0.84	....	1.03	....	11.2	....	2.8
	August 15	3	0.91	0.82	....	0.97	7.6	10.4	11.5	5.0
	July 25	2	0.92	0.84	0.76	1.00	8.1	6.9	14.2	0.4
	July 25	1	0.92	0.84	0.66	1.05	8.3	5.3	1.1	0.3
		Stump	0.92	0.85	0.66	1.11	8.3	10.5	1.1	0.2

Columns A and B under each heading refer respectively to values determined on the bark section at the time of isolation by ringing and on the same section after it had remained on the tree until mid-November.

and proportionally with the water-soluble protein content in excess of this amount.

When analytical values for insoluble protein nitrogen, for non-protein nitrogen, or for soluble sugars are similarly plotted against the hardiness, no

consistent evidence for an interdependence is obtained. The insoluble protein remains nearly constant at all degrees of hardiness. The non-protein nitrogen and the soluble sugars yield, in these plots, spot diagrams of such widespread variation that no dependence of hardiness on these components is manifested.

A series of analyses and hardiness tests run on the bark tissues of a number of normal (not ringed) trees during the transition period from August 1 to December 1, 1950, yielded a more intensive study of the relationship of hardiness to soluble protein in normal trees than that previously reported (**22**) and gave a striking confirmation of the reality of this inter-relationship. Two groups of normal trees were selected at the same locations (B and R) as the ringed trees, and the seasonal variations in hardiness and water-solu-

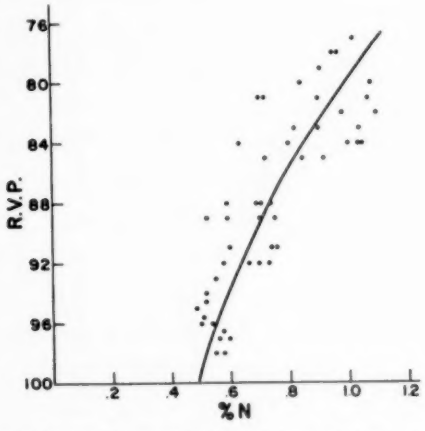

FIG. 1. Water-soluble protein content (percentage of dry weight of tissue present as water-soluble protein nitrogen) *versus* degree of hardiness (measured as the relative vapor pressure in the desiccation test which caused 50% killing of the cells of the tissue) of black locust bark tissue under conditions of normal seasonal variations (data from four trees) and under conditions of the ringing experiments (data from six trees).

ble protein content of the barks were followed in samples taken at frequent intervals throughout this period. Results obtained on representative trees from each of the groups are given in figure 2. It is evident from these graphs that the development of hardiness in one group of trees was considerably more rapid and took place at an earlier date than in the other group. It is remarkable how closely the rate at which the protein that accumulated in the tissues of these trees paralleled in each group the rate of development of hardiness. It is significant, also, that, when the degree of hardiness which is characteristic of most normal locust trees in winter is finally reached in both groups in November, the respective protein concentrations in the bark tissues of the two groups converge to approximately the same value. The same is true for the tissues of the terminal sections of the ringed trees where access to the leaves had permitted an almost normal development of hardi-

ness. No explanation can be given for the observed divergence in the rates and periods of hardiness development in the two groups of trees.

The indication given in figures 1 and 2 that only a part of the water-soluble proteins of the cells contributes to their hardiness raised the question as to whether the protein responsible for, or whose accumulation parallels, hardiness development might be identified and differentiated from the other soluble protein components electrophoretically. In November, large samples obtained by shredding off the living bark, as in the samples of 1949, were collected from logs corresponding to the sections of several of these trees ringed in 1950. These samples were extracted with water as described for the experiments of 1949, the extract was filtered to remove bark fragments

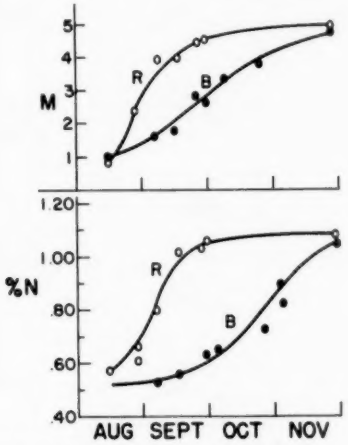

FIG. 2. Late summer and fall variations in hardiness (measured as molarity, M, of 1 : 9 :: $CaCl_2$: NaCl salt solutions required for 100% killing of cells in plasmolysis test) and in water-soluble protein content (percentage of dry weight of tissue present as water-soluble protein nitrogen) for two normal trees at two different locations (R and B). Dates refer to the times at which samples were taken.

and the soluble proteins were precipitated by saturating the solution with $(NH_4)_2SO_4$. The precipitate was filtered on a Buchner filter with suction, removed to cellophane sacs and dialyzed first against water and finally, after adjustment to approximately 1% protein concentration, against phosphate-NaCl buffer of 0.1 ionic strength and pH 7.1. Tiselius electrophoresis patterns were obtained after 8000 seconds electrophoresis against the equilibrium buffer at a field strength of 6.1 volts/cm. The conditions for electrophoresis used in the present experiments differ somewhat from those employed in the earlier studies (**2**). Buffer concentrations of 0.1 ionic strength are used here in contrast to 0.2 ionic strength buffers used earlier. A difference in the patterns appears in the absence of a fourth peak (D peak) in present patterns which was present in some of the patterns reported earlier.

In figure 3 are shown the descending boundary electrophoresis patterns

obtained under the above conditions with the water-soluble bark proteins from (a) typical, normal (not ringed) trees in June, August, November, and February, (b) the isolated sections, the stump, and the crown of the typical no. 4–R tree, and (c) the isolated sections, stump, and crown of the no. 1–B tree on which ringing was initiated so early in July that severe defoliation resulted. Three main peaks, denoting three electrophoretically distinct protein groups or entities, are present in all the patterns. Since the total pro-

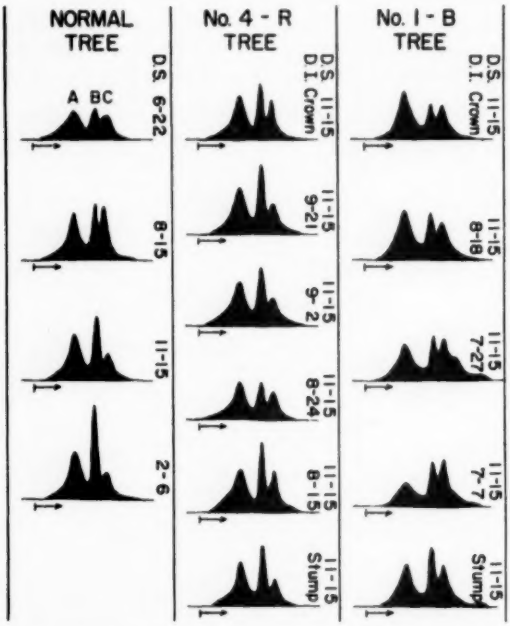

FIG. 3. Descending boundary electrophoresis patterns for the water-soluble proteins obtained from the living bark of black locust trees. Samples from normal trees were taken in June, August, November, and February. Samples from ringed trees no. 1–B and 4–R were collected in November from the various segments isolated at times described in text. Dates of sampling and of isolation of the segments on the ringed trees are indicated in the lines marked D.S. and D.I., respectively.

tein content of the solutions was adjusted to nearly the same value in each case, the areas under the peaks of the patterns yield only the comparative concentrations of the various groups of proteins present and not a comparison of their absolute concentrations in the bark tissues. The latter are indicated with fair accuracy in the analyses made on small samples as reported in tables IV and V.

Denoting the peaks as A, B, and C in their order of increasing mobilities under the conditions of the electrophoresis, it is seen from their areas that in the typical normal trees a large relative increase in the B fraction to-

gether with a relative decrease in the A and C fractions characterize the change from early summer to midwinter ratios of the fractions. The sharpness of the B peak (denoting close electrophoretic uniformity within the fraction) and the large increase in the relative area under the B peak in winter tissue as compared with summer tissue may be recognized as the most characteristic electrophoretic change in the water-soluble proteins which accompanies the wide change in hardiness of these tissues seasonally.

The patterns obtained with the water-soluble proteins of the various bark segments of the typical ringed tree (no. 4–R), by comparison with the patterns for the summer and winter proteins of normal trees, indicate that the ratios of the distinguishable fractions have approached, in November, the typical winter values. This change has occurred in the isolated segments independently of the time of isolation and even in those segments where no appreciable change in total water-soluble protein has occurred subsequent to the time of isolation. In other segments, those which were isolated later in the summer and in the stump and crown sections where the accumulation of water-soluble protein exceeded appreciably that present in early summer, it is found that even though the major accumulations occurred in the B fraction there was some increase in the total of A and C fractions as well.

The failure of the various bark segments of the no. 1–B tree to show this typical and preferential increase in the ratio of the B fraction (except in the stump section) may be taken to indicate that, even though a nearly normal increase in total water-soluble protein occurred in the late ringed sections, some factor normally derived from the leaves and required for synthesis of the B fraction was not available to the bark cells of this tree which had been radically, though not completely, defoliated in early July.

From these observations it is evident, (a) that a shift in the ratios of the electrophoretically distinguishable fractions of the total water-soluble proteins occurs as the winter season is approached whether or not there is an actual increase in such total protein, and (b) that, while generally the fraction B increases most markedly in relative amounts during the summer to winter adjustment, nevertheless, when any considerable increase occurs in total protein, some of this increase occurs in the A and C fractions as well as in the B fraction. While in the typical trees the B fraction increases most strongly as winter approaches (this same fraction is observed also to decrease most strongly in the spring) it is not possible to say that its concentration alone is the determining factor with respect to development of winter hardiness as distinguished from the effect of all of the water-soluble protein components. All fractions, when the total exceeds the summer minimum of the soluble proteins, appear to contribute to the hardiness of the cells.

When the extent of synthesis of water-soluble protein in the isolated sections of bark of the typical tree is examined in relation to the date of isolation of the sections, idealized curves as shown in figure 4 are indicated. In this figure, curve A represents the soluble protein nitrogen contents of the segments of bark at the date of their isolation from the crown by ringing,

and curve B represents their soluble protein contents after they have remained in place on the ringed tree until mid-November. Hardiness curves, which can be constructed by plotting the degree of hardiness (relative vapor pressure required for 50% kill of the tissue cells by desiccation) *versus* the date of isolation, parallel in contour and degree the increase in water-soluble nitrogen above the summer minimum.

From curve A of figure 4 it is evident that little actual accumulation of water-soluble protein above the minimum level of early summer occurs in the bark of these ringed trees prior to the latter part of August. Through September, synthesis and accumulation proceeds at an accelerating rate and reaches near a maximum by mid-November. Curve B indicates that, even though the amount of accumulated water-soluble protein may be low at the time that the segments are isolated throughout August, the capacity to synthesize is present in ever increasing degree. In a segment which is isolated

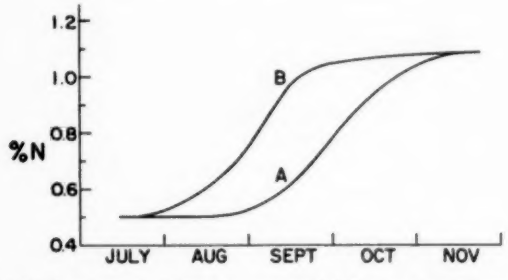

FIG. 4. Idealized curves showing the water-soluble protein content (percentage of dry weight of tissue present as water-soluble protein nitrogen) of the living tissue of the various isolated bark segments of typical ringed black locust trees, A, at the date of isolation of the segment, and B, after the isolated segment has remained in place on the tree until November. Dates on abscissa refer to the times at which the segments were isolated by ringing.

early in August not only is the amount of accumulated protein negligible but also the capacity to synthesize additional protein is very limited. By contrast, while the amount accumulated in a segment isolated in early September is still relatively small at the time of isolation, this segment will synthesize a considerable amount of protein by November, though not enough to confer maximum hardiness. Further, a segment isolated in late September, while containing at that time only a third to a half the amount of accumulated protein which will be characteristic of normal trees in November, has nevertheless acquired reserves sufficient to allow its protein content (and hardiness) to reach the maximum by mid-November.

Discussion

The results presented above and in earlier papers (**2, 22**) are consistent in that they indicate the existence of a direct correlation between the water-soluble protein content of the cells of the living bark of the black locust tree

and the ability of these cells to withstand injury by extracellular freezing or by dehydration. Under all normal and abnormal conditions for which the relationship has been tested, an accumulation of soluble proteins in the cells, in excess of a normal summer minimum equivalent to approximately 0.5% protein nitrogen, was accompanied by a proportionate increase in the winter hardiness of the cells. That fraction of the total water-soluble protein which corresponds to the summer minimum appears not to be appreciably effective in this function, although electrophoresis data indicate that some modification in the ratio of the components of the summer protein can occur in autumn in the early ringed sections, without an overall increase in total protein. Hardiness tests show that this change in ratio is accompanied by a small but detectable increase in hardiness of the tissue during that period. Until the exact mechanism is known by which these tissues avoid injury by extracellular freezing, it is not possible to state definitely that this relationship of water-soluble protein content to state of hardiness is causal but its occurrence in every case tested would suggest that it is so.

The synthesis of the protein which accumulates in the cells in excess of the summer minimum appears to be dependent upon the supply of some factor(s) derived from the leaves (or from reserves in the roots) by phloem transport. In typical trees this factor is nearly or completely absent from the bark cells until mid-August. After that time is begins to accumulate in the cells where it will allow subsequent synthesis of protein to take place, although this synthesis lags, in time, considerably behind the actual accumulation of the essential factor. The accumulation of this factor increases in rate, rapidly and concomitantly, with mobilization and translocation of leaf components in late August and September. In the case of only one tree (no. 3–R) did this factor appear to be retained in sufficient supply in early summer to allow for a nearly normal subsequent synthesis of protein in sections isolated at that time of year.

Analyses for the nitrogen fractions have failed to give a definite indication of the source of the nitrogen which, in a section isolated in September for example, becomes incorporated into the protein synthesized subsequent to the time of isolation. The insoluble protein fraction is definitely not a source since this fraction remains essentially constant throughout this period. Non-protein nitrogen compounds, which are present at the time of isolation, must be the source but analyses have failed to indicate any consistent decrease in this nitrogen fraction as the water-soluble protein increases in amount. A more detailed analysis of this nitrogen fraction is called for. It is possible that non-protein nitrogen containing compounds (other than the essential factor) may diffuse into or out of the water conducted in the xylem past the isolated section of bark and thus render the analyses meaningless in this regard. The essential factor which limits the soluble protein synthesis, however, can reach the tissues only through the phloem.

Evidence from electrophoretic analysis of the water-soluble proteins of the various bark segments of ringed trees suggests that, when this essential

factor is available, synthesis of a particular protein fraction or component (peak B) occurs preferentially. It is of interest that this particular protein fraction disappears preferentially in spring with leaf emergence. This points to the probability that this protein fraction serves a double function of promoting hardiness in the bark cells and as a storage protein for factors which are essential to leaf development in spring and leaf function in summer.

Because of the observed relationships of water-soluble protein content to hardiness, of the effects of ringing on hardiness and on protein content, and of the time when the maximal effect of this ringing is exerted, it appears that the hardening process, in the black locust at least, is linked directly to the process of autumnal migration. This is the well known phenomenon observed in trees prior to leaf fall when an evacuation of nitrogenous and other substances from the leaf occurs, these being translocated in some form to the bark of the twigs, trunk and roots where they are stored in a form which, in the case of nitrogen, appears to be water-soluble protein. Many reports exist in the literature which indicate that accumulation of protein occurs in the bark in winter. In other instances an evacuation of nitrogen from the leaves prior to abscission has been shown. As early as 1923 KERN (**9**) reported that highly refractive bodies accumulate in the cells of tree bark in winter and that these bodies give positive microchemical tests for proteins. RIPPEL (**19**) in 1921 and COMBES (**3**) in 1925 showed that nitrogen migrates from the leaves in fall and accumulates in the branches and trunk of the tree. Others (**8, 14, 15, 17, 18, 25**) have reported the occurrence of this phenomenon. LOOMIS (**13**) in the course of certain ringing experiments, demonstrated accumulation of protein in the bark of trees in winter. JONES and PHILLIPS (**7**) observed that there is more protein in the bark of the black locust in winter than in summer. TRAUBE (**26**), on the basis of an observed increase in non-amino organic nitrogen in the cortex and phloem of apple trees in winter, inferred that there must be an increase in proteins. The exact nature of the protein stored in the bark has, however, remained obscure and because of inadequate extraction and characterization procedures, it has been identified commonly as insoluble protein.

The translocation and accumulation of carbohydrates, in contrast to that of nitrogen, does not seem to be a process that is peculiar to autumnal migration. In a detailed study of seasonal carbohydrate variations in the black locust (**24**) it was found that accumulation of carbohydrates in bark tissues begins immediately after the slowing down and cessation of active growth of the tree in midsummer and that the accumulation which is observed during autumnal migration is simply a continuation of this process. Thus a considerable increase in carbohydrate content long precedes the period during which maximal increase in hardening occurs.

It is surprising that the significance of the autumnal migration of nitrogen and of its accumulation in the form of water-soluble protein in the bark in relation to frost hardening has not hitherto been recognized, particularly in view of its coincidence, at least in the locust, with the period of maximal

hardening. LEVITT (**12**) summarized the evidence concerning the relation of nitrogen to hardiness by stating that there does not appear to be any such relationship. Furthermore it is stated that hydrolysis of protein to simple nitrogen compounds is a characteristic of the hardening process in plants. The evidence presented in this paper indicates, in contrast to these statements, that autumnal migration provides not only reserves to the bark for growth in spring but also nitrogen compounds essential for synthesis of water-soluble proteins which are concerned directly with the mechanism of hardening.

Any discussion of the mechanism whereby the increase in water-soluble protein in the cells contributes to their frost hardiness is quite premature. If an hypothesis is desired as to a possible mode of their action, it may be postulated that they promote hardiness (a) by promoting the capacity of the protoplasm to undercool, thus retarding any tendency for ice formation to occur intracellularly prior to extracellular ice formation, (b) by acting as plasticizers or mechanical buffers in the cell, as its volume is decreased by dehydration due to water removal to the extracellular ice crystals during freezing, thereby acting to retard irreversible disruption of cellular organization. That these proteins contribute to the high non-solvent space of the cells was first observed by LEVITT and SCARTH in hardy cells (**10**). In view of the mechanical nature of frost injury, resistance to such injury could easily be enhanced by an increase in non-solvent space. ASAI (**1**) reports a high non-solvent space in bark cells of hardy roses. An increased protein content may also increase hardiness by augmenting the hydrophily of the protoplasm (**18**). It is not suggested here that the chemical mechanism of the hardening process is limited to the effect of accumulation of water-soluble protein. There is evidence that other products of the degradation and hydrolytic processes occurring in the leaf cells before leaf fall are translocated to the bark. A loss of phospholipids in the leaves as well as a migration of phosphorus in some form to the bark has been reported (**4**) to occur at this time. There is evidence of an increase in the total lipid in the bark in winter (**7**). Together these observations would suggest that the protein synthesis may be only a part of what amounts to a net synthesis of new protoplasmic materials within the cell to render it hardy. It is difficult to escape the conclusion, after visual observation and micromanipulation of hardy and non-hardy cells of the same plant, that there is a greater concentration of protoplasm and of protoplasmic particles in hardy cells. It is possible that hardiness is promoted by the total effect of the increased protoplasmic concentration rather than by the higher protein concentration alone.

The available evidence does not suggest the participation to any considerable extent of the soluble carbohydrates in the mechanism of hardiness. Obviously a minimal level of carbohydrate must be maintained as an energy source for the normal metabolism or respiration of the bark cells. A too early or considerable defoliation or ringing of trees may affect hardiness in this manner. It is also possible that very high concentrations of sugars may contribute somewhat to the attainment of extremes of hardiness but their

role in this regard has not yet been discerned. In connection with the present studies it has been observed that high concentrations of starch in cells will render them more susceptible to frost injury. Fluctuations in starch content may result in detectable apparent changes in frost hardiness.

Of significance is a small but appreciable increase in hardiness which occurs in the early ringed sections from the time of ringing until November even without a detectable change in total protein content. A subsidiary mechanism of hardening which is independent of the supply of the substances derived from the leaves may exist. This might be of the nature of a change in the physical structure of the protoplasmic membrane which has been observed to occur during hardening (**11, 20**). The electrophoretic analyses also suggest that there may occur some conversion of protein without a net synthesis which would result in a small increase in its protective action against frost injury to the cell.

Summary

Segments of the bark of black locust trees were isolated, *in situ*, from phloem continuity with the rest of the tree by ringing. The rings were cut at different times along the trunks from July to September thus yielding a series of segments on each tree, all being isolated from the root at the time the first rings were cut and each being isolated from the crown only after a later ring was cut above that particular segment. Injury to the xylem during ringing of the bark was avoided.

The living bark tissues from the various segments were tested for winter hardiness and were analyzed for insoluble protein nitrogen, water-soluble protein nitrogen, non-protein nitrogen, soluble carbohydrate and starch contents at the time of isolation and again in November. A positive correlation was found to exist between the water-soluble protein content (in excess of a summer minimum) and the degree of hardiness of the tissues under all conditions studied. No such relationship was indicated for the other components for which analyses were made.

Synthesis, in autumn, of the water-soluble proteins is dependent upon the prior accumulation in the bark tissue of some factor(s) which must reach the bark cells through phloem transport before their isolation, by ringing, from the leaves (or root) of the tree. This factor appears to be mobilized from the leaves only in late summer and autumn, reaching maximal transport in September just prior to leaf abscission.

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FLORAL INDUCTION AND DEVELOPMENT IN ORCHARD GRASS[1]

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PETERSON and LOOMIS (**11**) found that both low temperature and short day lengths were necessary for floral induction in Kentucky bluegrass, but growth and development of flowers were favored by long days and a moderately warm temperature. Other investigators (**8, 9**) have obtained similar results with smooth brome grass. COOPER (**2**) found that perennial rye grass remained vegetative under continuous illumination in a warm greenhouse, whereas annual and Italian types flowered well under these conditions.

From a physiological viewpoint, less emphasis has been given to initiation of floral primordia in perennial grasses than to induction. However, detailed morphological studies on the seasonal development of the shoot apices have been conducted on a number of species (**2, 7, 12, 13**), including orchard grass (**4**). In general, investigators agree that the growing points remain in a vegetative condition throughout the fall and winter and initiate floral primordia the following spring with increasing day-length and temperature.

The production of mature flowers and fruits from the floral initials is likewise favored by long days (**1, 3, 5, 15**), although certain species from southern latitudes may require short days (**10**). At this later stage, orchard grass is a long-day plant with a lower critical limit of approximately 12 hours (**1, 15**). The production of flowers and fruits is accomplished in a short period of time after initiation, and requires optimum growth factors such as available nitrogen and photosynthate.

Orchard grass, *Dactylis glomerata* L., which has been observed to produce abnormally low yields of flowers and seed under certain environments, was used in this study. The effects of photoperiod, temperature, and their interrelationships in induction, initiation, and further development of flowers have been considered separately and collectively. Floral induction is defined as a chemical or hormonal differentiation resulting from the fulfillment of certain thermo-photoperiodic requirements. Floral initiation is the morphological transformation of an induced growing point from a vegetative to a floral primordium. Further floral development results in the production of macroscopic flowers.

Methods and materials

Uniform sods of various clones of vegetative orchard grass growing in six-inch pots were used as the experimental material and were exposed to

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combinations of temperature and photoperiod during the induction period, and then transferred to long-day conditions known to be favorable for flowering to determine the degree of induction obtained. Or plants were exposed to conditions known to be favorable for induction, and were then given varying treatments during the initiation and flowering periods to determine the limiting conditions for these development phases.

Three local strains of orchard grass were used: OG–126, free-flowering, early strain; OG–144, free-flowering but late strain; OG–14, weakly flowering, early strain. Photoperiods included normal fall or spring days, which varied from 12 hours on September 22 to 9 hours and 7 minutes on December 22, and up again to 12 hours in March and 14 hours at the end of April. Constant photoperiods of 9 to 20 hours were obtained by covering plants morning and evening, or by supplementary incandescent light of 100 to 150 fc. Temperatures employed in the study were normal fall temperatures ranging from 80 to 0° F, thermostatically controlled greenhouse temperatures of 65 to 75° F, and temperatures which varied from 60° F on sunny days to moderately sharp freezing in low temperature rooms and unheated or partially heated greenhouses.

Initiation of inflorescence primordia was observed by dissection of fresh or preserved growing points. Induction was determined by observing subsequent initiation or development of flowers on treated sods.

Results

Floral induction

The first induction experiment was run in the fall of 1949 with the three strains of orchard grass, two temperatures (outdoor and warm greenhouse), three photoperiods (nine hours, normal fall photoperiod, and 18 hours), and four replications. Treatments were started on October 10 with established plants in pots and continued until December 23, when all plants were transferred to a warm greenhouse and 18-hour photoperiods. Plants maintained outside were frozen back in December, while plants in the warm greenhouse were growing vigorously.

Panicles began to appear approximately three weeks after transfer to the warm greenhouse. Of a total of 115 panicles produced, all but one were from plants which had been held during the induction period with nine-hour or normal fall photoperiods at the cool, normal fall temperature. Only one panicle developed on plants held at 65° F. Plants maintained under 18-hour photoperiods did not flower regardless of the temperature. Significantly more inflorescences were produced by plants held with nine-hour photoperiods throughout the induction period. These plants were exposed to more short days than the checks, but they were also protected at night by covers and showed less freezing injury. Either or both factors may have been active. Of the three strains used, strain 144 consistently produced more panicles than strains 126 and 14, and differences between strains were highly significant.

TABLE I

NUMBER OF FLOWERING HEADS PRODUCED BY FOUR GRASSES AFTER INDUCTION UNDER DIFFERENT TEMPERATURES AND PHOTOPERIODS FROM OCTOBER 9 TO DECEMBER 22, 1950. FLOWERS DEVELOPED IN A WARM GREENHOUSE UNDER 18-HOUR PHOTOPERIOD AFTER DECEMBER 22 (DATA ARE PANICLES ON FOUR POTS).

Type of grass	Strain or variety	Length of photoperiod during induction, in hours									
		9	11	12	12.5	13	13.5	14	16	18	N
						Cold greenhouse					
Orchard grass	OG-126	34	33	38	17	1	0	0	0	0	18
	OG-144	32	36	45	8	8	1	3	0	0	14
	OG- 14	35	23	28	14	6	0	4	0	0	13
	Total	101	92	111	39	15	1	7	0	0	45
Kentucky bluegrass	Commercial	26	41	33	14	8	5	3	0	0	51
						Warm greenhouse					
Orchard grass	OG-126	0	0	0	0	0	0	0	0	0	0
	OG-144	0	0	0	0	0	0	0	0	0	1
	OG- 14	0	3	0	0	0	0	0	0	0	0
	Total	0	3	0	0	0	0	0	0	0	1
Kentucky bluegrass	Commercial	0	0	0	0	0	0	0	0	0	0

The 1949 experiment was repeated in 1950 with 10 photoperiods, three temperatures and five replications. Plants exposed outside in pots were killed by November temperatures of – 11° F, so that the comparison shown is between a warm greenhouse and a cold greenhouse in which minimum temperatures for November and December averaged about 40° F, and readings of 20° F were recorded. The normal-day plants were most exposed, and showed freezing injury to the leaves which may have accounted for their poorer flowering.

The data obtained in 1950 (table I) are in close agreement with those obtained in 1949. As was found in 1949, floral induction did not occur in a warm greenhouse regardless of the photoperiod used. At the low tempera-

TABLE II

ANALYSIS OF VARIANCE OF A PART OF THE DATA ON ORCHARD GRASS FROM TABLE I, INCLUDING VARIOUS PHOTOPERIODS IN THE COLD GREENHOUSE.

Source of variation	Df.	Ms.
Photoperiods	4	67.25*
(9 to 12-hour *vs.* normal)	1	140.45*
(9-hour *vs.* normal)	1	70.54*
(12-hour *vs.* 9-hour)	1	13.34
Replications (within photoperiods)	20	7.23
Strains	2	16.12
Strains × photoperiods	8	4.45
Error	40	4.44

*F value exceeds 1% level of significance.

ture, good flowering was obtained from all strains that were subjected to photoperiods of nine to 12 hours, including normal fall day. The greatest number of flowering heads resulted from the exposure to a 12-hour photoperiod, but differences between 9, 11, and 12 hours were not significant, as can be seen in table II. Lengthening the photoperiod as little as one half hour from 12 to 12.5 hours reduced flowering by approximately two thirds,

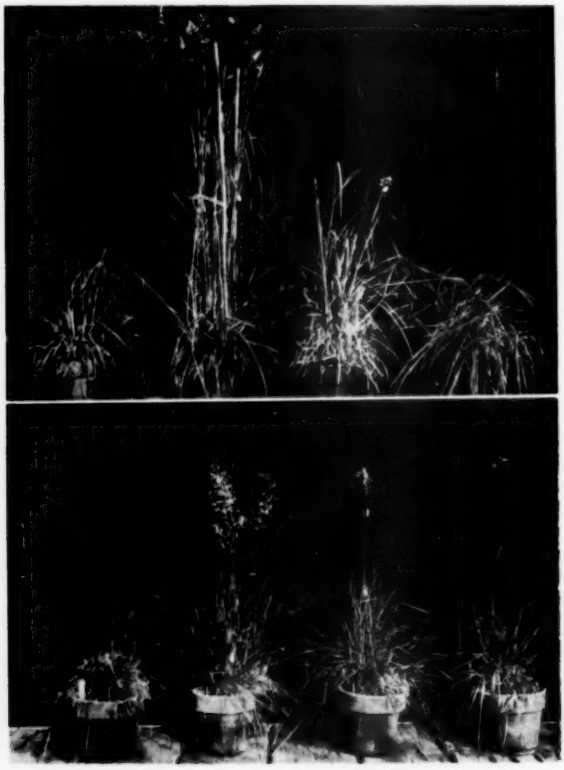

FIG. 1. Response of orchard grass (above) and Kentucky bluegrass (below) to induction treatments as follows (left to right): warm, 12-hour day; cool, 12-hour day; cool, 14-hour day; cool, 18-hour day.

a highly significant difference. Only scattered flowering occurred in plants induced with day lengths longer than 12.5 hours.

Kentucky bluegrass, reported to be exacting in induction conditions (**11**), was included in this experiment for comparison. The data obtained are similar to those from orchard grass in that of a total of 181 panicles produced, all were from plants held during the fall at a low temperature, and all but 30 panicles were from plants that received photoperiods of 12 hours or less (table I). The typical flowering response of both orchard grass and

Kentucky bluegrass is shown in figure 1. In general, the time required for appearance of flowers on induced plants of both grasses was shortest with the most effective induction treatments. Plants receiving short days at a low temperature formed panicles profusely and rapidly when transferred to favorable growing conditions.

Growing points were collected at the conclusion of the induction treatments in 1949, and were studied for morphological evidence of floral development (cf., fig. 5). No microscopically visible floral primordia were found in a total of 46 growing points collected from sods maintained at the normal fall temperature, yet plants induced under normal fall and nine-hour photoperiods flowered profusely when transferred to a warm greenhouse for development. Growing points from all plants maintained at 65° F were vegetative also, but these plants did not flower when transferred to conditions favorable for floral development. The presence of a ripe-to-flower condition in these plants without any morphological expression of floral development is evidence for the production of a specific flowering substance or reaction. The processes resulting in the formation of this flowering condition are defined as floral induction.

Natural induction

Orchard grass in the field is exposed to cool days with a shortening photoperiod in the fall and to warmer days with a lengthening photoperiod in the spring. Normal induction may be assumed to occur during one of these periods. Tests of natural fall induction were run by transferring sods of strain 14 to a warm greenhouse at 15-day intervals. These remained vegetative indefinitely when transferred before November 1. All plants flowered when transferred on or after November 1, with the best response from the December 15 lot. Essentially the same results were obtained in 1950, using potted sods plunged in the field for protection. Again, plants transferred before November 1 remained vegetative while those transferred November 1 or later flowered (table III). Flowering of plants transferred in December 1950, was reduced by freezing injury during severe weather in late November. Potted sods in the more exposed positions were killed, while others recovered but the induced growing points were injured and new growth from axillary buds was strictly vegetative.

TABLE III

NUMBER OF FLOWERING HEADS DEVELOPING IN ORCHARD GRASS AFTER TRANSFER FROM NATURAL FALL CONDITIONS TO A WARM GREENHOUSE AT DIFFERENT DATES IN 1950.

Strain	Date of transfer						
	September 15	October 1	October 15	November 1	November 15	December 1	December 15
OG–144	0	0	0	21	13	1	3
OG–14	0	0	0	27	19	15	0
Total	0	0	0	48	32	16	3

Natural spring induction was studied by transferring vegetative, potted plants of orchard grass to the field in March and April. Plants transferred early were injured by freezing, and those moved from the greenhouse in April flowered sparsely or not at all because the 13- to 14-hour photoperiod was too long for induction.

In the spring of 1950, potted orchard grass and bluegrass plants were transferred to an unheated greenhouse under normal photoperiods. Transfers were made from warm, long days to cold, spring days at approximately two-week intervals starting February 20. Flowering responses, average minimum temperatures and day lengths are shown in figure 2. Vigorous

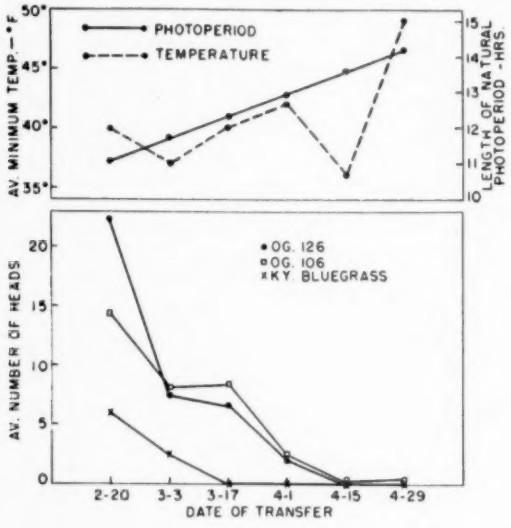

FIG. 2. Average number of flowering heads per pot produced by orchard grass and Kentucky bluegrass in relation to the average minimum temperature and average day length when transferred to spring conditions on different dates.

flowering resulted from the first transfer of orchard grass, with an average minimum temperature of 40° F and an average day length of 11.05 hours during the first two weeks after transferring. Strain 126, a heavy flowering strain, produced over 22 panicles per pot, while strain 106, a weak flowering strain, produced 14 panicles per pot. This first transfer to a cold greenhouse under relatively short photoperiods is believed to have reflected the genetic flowering potential of the two strains. The number of flowering heads was significantly reduced in the second transfer two weeks later. The average minimum temperature was lower, but the average photoperiod of the next two weeks had increased to about 12 hours. A few panicles were obtained from plants transferred on April 1, but plants transferred on or after April 15 remained vegetative. Representative plants of orchard grass from the

various transfers are shown in figure 3. The day length on April 15 is approaching the 14 hours which was found to be too long for induction (table I). It is of interest to note that orchard grass generally does not break dormancy until about April 15 in this latitude, precluding natural spring induction in strains of the type used here.

Kentucky bluegrass flowered rather weakly when transferred to spring conditions on February 20 and March 3, but remained vegetative with subsequent transfers. These experiments show that the photoperiodic responses of orchard grass and bluegrass are determined by the effective day length during the induction period and are independent of the effects of shortening fall or lengthening spring days. The poorer flowering of bluegrass (fig. 2)

TABLE IV

FLOWERING RESPONSES OF SOME ECONOMICALLY IMPORTANT GRASSES SEEDED IN THE FIELD IN EARLY SPRING OF 1950.

Grass	Flowering responses in 1950
Orchard grass *(Dactylis glomerata)*	No flowering
Reed canary grass *(Phalaris arundinacea)*	No flowering
Redtop *(Agrostis alba)*	No flowering
Kentucky bluegrass *(Poa pratensis)*	No flowering
Red fescue *(Festuca rubra)*	No flowering
Crested wheat grass *(Agropyron cristatum)*	No flowering
Tall fescue *(Festuca elatior)*	Sparse flowering
Perennial rye grass *(Lolium perenne)*	Sparse flowering
Northern smooth brome grass *(Bromus inermis)*	Light flowering
Southern smooth brome grass *(Bromus inermis)*	Light flowering
Mountain brome grass *(Bromus marginatus)*	Light flowering
Tall oat grass *(Arrhenatherum elatius)*	Medium flowering
Timothy *(Phleum pratense)*	Medium flowering
Canada wild-rye *(Elymus canadensis)*	Heavy flowering
Big bluestem *(Andropogon furcatus)*	Heavy flowering
Side-oats grama *(Bouteloua curtipendula)*	Heavy flowering
Weeping love grass *(Eragrostis curvula)*	Heavy flowering
Switch grass *(Panicum virgatum)*	Heavy flowering

is considered to be due to the necessity of more short days for induction in this species. Day lengths were favorable for induction on March 17 (cf., table I), but became too long before induction could be completed.

Flowering responses of orchard grass and other grasses, spring seeded in the field, corroborate the findings of the above experiment. Orchard grass, and other cultivated grasses of the north temperate zone, generally remained vegetative throughout the season of seeding (table IV). An exception was found in timothy which produced flowers and fruits rather abundantly in the seeding season, and to a lesser degree in smooth brome grass which flowered sparsely with variable responses from northern, southern, and mountain types. The response of orchard grass was absolute, reflecting the requirement for a shorter photoperiod than was afforded by natural spring conditions at the time the seedlings were mature enough to be receptive.

FIG. 3. Flowering response of orchard grass transferred to spring conditions on the following dates (left to right): February 20; March 3; March 17; April 1.

Many American native grasses and introduced grasses indigenous to southern latitudes flowered profusely by the end of the seeding season, appearing to be independent of low temperature and short photoperiod requirements.

LOCALIZATION OF INDUCTION

During the course of this research, hundreds of pots of orchard grass sods, set outside for natural fall induction, were injured by severe freezing. Some were killed outright, but a typical reaction was a reversion to vegetative growth. The main growing points, from which inflorescences normally develop, did not show injury at first, but did not make further growth, either

FIG. 4. Localized effect of photoperiod on orchard grass. All plants were in a cold greenhouse, but shoots left of divider received normal fall day; those right of divider an 18-hour day.

TABLE V

FLOWERING OF ORCHARD GRASS AFTER INDUCTION WITH VARYING COMBINATIONS OF DAY LENGTH AND TEMPERATURE (DATA ARE PANICLES ON FOUR POTS).

Pretreatment—warm for three weeks	Posttreatment—cool		
	Long day, three weeks	Short day, two weeks	Short day, three weeks
Long day	0	0	36
Short day	32	....	38
Short day	26	16	....
Short day + 1 week warm, long day	....	1	..
Short day + 2 weeks warm, long day	...	0	...

vegetative or floral, and eventually became necrotic. Lateral buds developed under these conditions and none of these developed flower buds. We may assume, either that floral induction was destroyed by the freezing, or that induction was localized in the apical growing points which were injured enough to arrest further development, thus preventing flowering.

Clonally propagated sods of orchard grass, such as were used here, are vegetatively connected throughout so that a flowering hormone should be able to affect the entire sod if it were systemic in its actions. Individual sods were held in a cold greenhouse to test this hypothesis, with half of the sod exposed to a long day and the other half to a short day. The results, illustrated in figure 4, show that induction was strictly localized, and suggest, in connection with the freezing experiments, that it was further limited to growing points in a specific physiological condition.

Interaction of photoperiod and temperature

In the previous experiments the plants were exposed simultaneously to the actions of day length and temperature. The results indicated that an exposure for three weeks to a photoperiod of less than 12.5 hours and an average minimum temperature of about 40° F was adequate for induction of orchard grass.

A number of experiments were performed in which the short-day and

TABLE VI

FLOWERING OF ORCHARD GRASS AFTER INDUCTION WITH VARYING DURATIONS OF SHORT DAYS AND LOW TEMPERATURE.

Pretreatment of warm, short days	Posttreatment of cold, long days	Panicles
1 week	3 weeks	8
2 weeks	3 weeks	14
3 weeks	3 weeks	15
3 weeks	1 week	1
3 weeks	2 weeks	4
3 weeks	3 weeks	12

cool-temperature treatments were separated. Pertinent data from these experiments are summarized in tables V and VI. Perhaps the most significant point in these data was the effectiveness of long, cold days following short, warm days, thus showing that the photo- and thermal-induction reactions could be separated. The limit of three weeks of cold, short days, previously established, appears to be set by the thermal reactions. Two weeks of cold, short days were less effective than three weeks of cold, long days following warm, short days (table V), and cold posttreatments of two weeks were relatively ineffective, whereas two weeks of warm, short-day pretreatment were as effective as three weeks (table VI). If the cool temperature exposure preceded the short photoperiod, no induction occurred. Also, one or two weeks of long, warm days interposed between short-day and cool treatments prevented induction. These data suggest that the photoperiodic reaction is the basic one, and that the low temperature effect is then necessary to complement the short-day reactions and insure induction.

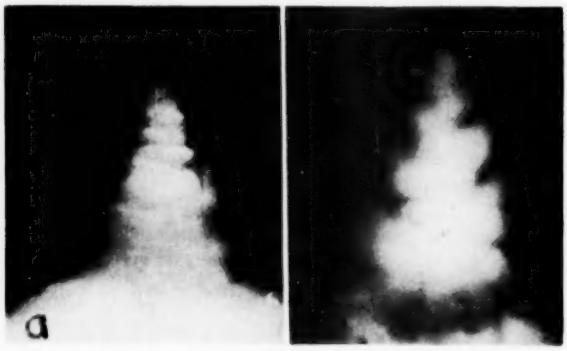

FIG. 5. Morphological development of growing points: A. Vegetative growing point as shown by check or induced plants. B. Floral primordium initiation after three to five long days with warm temperature. (50×.)

FLORAL INITIATION AND DEVELOPMENT

The first experiment on initiation failed because of cold injury to the induced growing points, and it was necessary to induce a second lot of plants in a cold greenhouse. At the end of the induction period in December, all growing points on these plants were vegetative (fig. 5 A). Five induced sods of clone OG–144 were transferred to each of five light treatments in a warm greenhouse and observed for initiation of inflorescence primordia and for flowering. Distinct inflorescence primordia (fig. 5 B) were observed in plants under a 24-hour day three days after transfer to this photoperiod at a warm temperature, but morphological evidence of flowering was not apparent in plants under 16-hour, 13-hour, normal winter day (9 to 10 hours), or continuous darkness, as can be seen in table VII. After 12 days, all of the main shoots of the plants under a 24-hour day which were observed had pro-

TABLE VII

FLORAL INITIATION AND DEVELOPMENT IN INDUCED ORCHARD GRASS PLANTS GROWN UNDER DIFFERENT PHOTOPERIODS IN A WARM GREENHOUSE.

Duration of treatment	Length of photoperiod	Number observed	Developmental stage			Average length of apex
			Floral	Elongated	Vegetative	
days						*mm.*
3	Continuous light	10	3	5	2	.86
	16 hours	10	0	1	9	.37
	13 hours	10	0	0	10	.35
	Normal day	10	0	0	10	.38
	Continuous darkness	10	0	0	10	.35
12	Continuous light	10	10	0	0	2.80
	16 hours	10	9	1	0	1.30
	13 hours	10	4	3	3	.60
	Normal day	10	0	0	10	.36
	Continuous darkness	10	0	0	10	.37
37	Continuous light	....	Flowering			
	16 hours	....	Flowering			
	13 hours	....	Jointing*			
	Normal day	7	0	2	5	.45
	Continuous darkness	10	0	0	10	.36

*Decumbent stems with panicles enclosed in a leaf sheath.

duced well developed inflorescence primordia averaging 2.8 mm. in length. Of 10 growing points from plants under a 16-hour day, nine were floral and one had elongated in the transition from vegetative to floral primordium. The average length of the apices observed after 12 days under this photo-

FIG. 6. Floral development of induced plants after 37 days. Left to right, day lengths of 9 to 10, 13, 16, and 24 hours.

period was 1.3 mm. Almost equal numbers of floral, elongated, and vegetative apices were obtained after 12 days under 13 hours. Only vegetative growing points with an average length of 0.36 mm. were observed in plants under days of 9 to 10 hours or continuous darkness. After 37 days, plants under days of 24 and 16 hours had formed well developed panicles. Plants under days of 13 hours were jointing or had elongated stems with panicles enclosed in leaf sheaths (fig. 6). Except for two elongated apices, all of the plants under winter days and continuous darkness were vegetative. The continuous dark plants were colorless and etiolated.

Plants from the normal winter day and continuous darkness treatments were transferred to a 24-hour photoperiod at a warm temperature after 45 days. The short-day plants soon began to produce panicles and flowered normally, even though floral development had been arrested for 45 days by

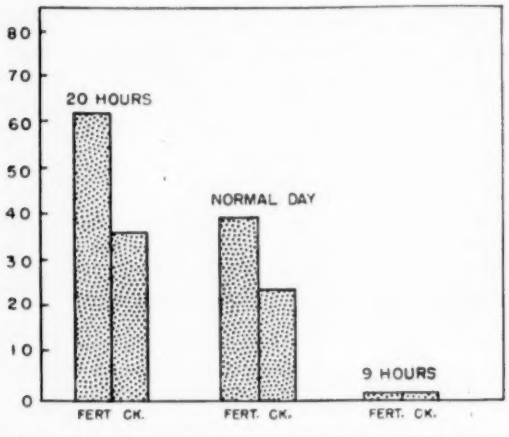

FIG. 7. Number of heads produced by orchard grass subjected to three photoperiods and two fertility levels (average of two strains; normal days in May).

the adverse photoperiods. Plants from continuous darkness regained their color and resumed growth but failed to flower, indicating a loss of induction, perhaps because of the long period of inadequate carbohydrate nutrition.

FURTHER FLORAL DEVELOPMENT

The effect of photoperiod on the further development of initiated panicles was marked (fig. 7). Plants under 20-hour photoperiods produced 56% more panicles than those under normal spring days of 14 to 15 hours. Only a few heads were finally produced by one strain under days of nine hours, and these were abnormal. Differences between the three photoperiods were significant, but differences between days of 20 hours and normal day were not.

An initial complete fertilization, followed by supplementary nitrogen fertilization, increased flowering in all plants under days of 20 hours and

normal days, but it had no effect on plants under days of nine hours. Differences between levels of fertility were not statistically significant; however, these differences were fairly large and consistent in both photoperiods and were believed to be real. Data for plants under days of nine hours were mostly zeros and were not included in the analysis, leaving limited degrees of freedom and increasing the difference required for significance.

It should be noted that plants in this experiment were subjected to the various photoperiods after initiation of floral primordia (fig. 5 B), and the failure to flower was a result of the inhibition of the young, developing inflorescences. Dissection of several main shoots from nine-hour plants revealed that many of these inflorescences had aborted.

Discussion

The work with orchard grass and bluegrass emphasizes a point that is frequently overlooked in studies of photoperiodism. The requirements of

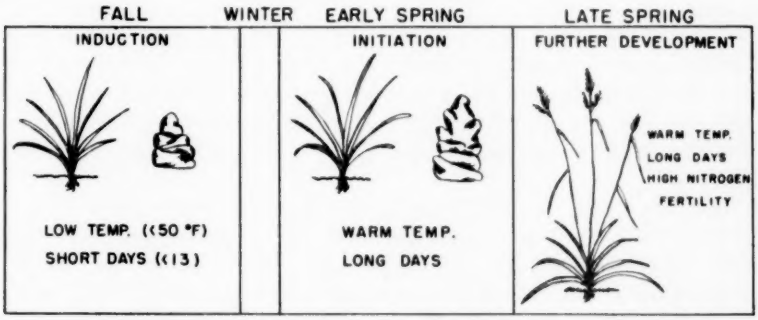

FIG. 8. Diagrammatic representation of flowering in orchard grass.

the three principal phases of flowering, induction of the flowering condition, initiation and early growth of the inflorescences, and complete development of the flowers, may be very different. In the strains of these grasses used in this work, induction requires photoperiods on the order of 12 hours or less, either accompanied or immediately followed by temperatures of 5 to 10° C. The initiation of floral development requires warmer temperatures and somewhat longer days, with slow initiation on 13-hour days. Normal development of panicles requires moderate temperatures and photoperiods of more than 13 hours (fig. 8).

The conclusion that both short day and low temperature are required is supported by the natural induction experiments in the fall of 1949 and 1950. Plants transferred from natural conditions to a warm greenhouse remained vegetative if transferred before November 1, whereas, plants transferred on or after this date flowered in both years. The temperature was generally cool during October, especially at night, but it is unlikely that the temperature was similar enough in both years to cause this consistent response.

Rather, it would appear that a sufficient exposure to the day lengths, constant for both years, that had decreased into the effective range, was the limiting operative factor. The necessary exposure to short days was effected by November 1 along with enough cool weather to insure induction.

Low temperature prevails in the spring while plants are physiologically active, but we were unable to obtain spring induction in field plantings under natural conditions in Iowa. Orchard grass and several other grasses failed to flower when seeded in early spring. The temperature on April 15, the time plants normally break dormancy at this location or become active from seedings, is generally cool, but the day length is 13 hours and 21 minutes, which is too long for successful induction. Other grasses were less exacting for the low-temperature, short-day requirement and flowered sparsely, while others such as timothy flowered abundantly. These responses reflect genetic variability between and within species of the same geographic origin. The American native and southern grasses tested were independent of this requirement, as they flowered heavily in the seeding year, while the European economic grasses tended to be fall induced. There were exceptions to this rule, however, as in *Bromus inermis* and *Arrhenatherum elatius*, which suggest that spring or summer induced strains might be isolated by selection. We might postulate that Mediterranean grasses, or species developed in regions of open winters and dry summers, have been naturally selected for winter induction, maximum earliness of flowering and no exhausting, late flowering.

Kentucky bluegrass and orchard grass flowered if vegetative sods were transferred to a cold greenhouse and late winter photoperiods, but these grasses ceased to be induced after March 3 and April 1 respectively. Apparently bluegrass must be exposed to effective short days for a longer period than orchard grass, as both species appear to have approximately the same upper limit. The fact that they normally resume growth in the spring around April 15 precludes the probability of natural spring induction in these species in Iowa, and the belief that induction resulting from fall conditions is lost over winter and regained in the spring is not tenable. Not only was fall induction found in dormant plants transferred to flowering conditions during the winter, but induced plants were held in vegetative growth with short photoperiods in a warm greenhouse for 45 days, and then flowered freely when the days were lengthened to the level required for initiation and development of inflorescences.

The presence of a ripe-to-flower condition without morphological evidence of floral development indicates that induction is chemical in nature, and may be classed as the formation of a flowering hormone, although the possibility of a complex, flowering organization of the protoplasm is not excluded.

Short days and low temperature normally occur simultaneously; however, the two factors could be separated, with all short days given at warm temperature provided they preceded the low temperature exposure. The

fact that the short-day stimulus must come first suggests that the low temperature in some way acts on the short-day stimulus in a stabilizing or modifying capacity. A week of warm, long days destroyed the short-day stimulus before it could be stabilized by low temperature.

The formation of floral primordia in induced orchard grass occurs normally in the spring, coincident with longer day lengths and warm temperature. No morphological evidence of floral development could be found after 37 days under normal winter days (9 to 10 hours) or continuous darkness, even though the temperature was optimum, but initiation and flowering occurred rapidly if induced, short-day plants were transferred from the adverse photoperiods to long days. The continuous-dark plants were etiolated and too depleted in photosynthate for flowering. The short-day treatments arrested development without losing or reversing induction, even though the temperature was warm. How long induction could be held by short days alone is open to conjecture, but presumably it could be held indefinitely by both low temperature and short days, as is done naturally over winter.

Further development of flowers involves rapid growth of flower buds into expanded panicles on elongated stems. This rapid expansion requires optimum growth factors of moderate temperature, ample water, nitrogen, and carbohydrates. Long days are favorable for development, but this phase may be less critical in its photoperiod requirements than induction and initiation. When plants with initiated inflorescences were allowed to develop with nine-hour photoperiods, many shoots showed tendencies of flowering without producing visible panicles. Dissection of such shoots showed abortion of the initiated inflorescences, perhaps as a result of inadequate photosynthate in these plants which were under short days at a high greenhouse temperature. For maximum seed yield, agronomic practices must be directed at this phase of flowering to supply optimum growth factors. Water is usually adequate in the spring when these grasses flower, but marked responses are obtained from nitrogen, especially from spring applications (**6, 8, 14**).

Summary

The effect of photoperiod and temperature and other conditions on induction, initiation and further development of flowers in three strains of orchard grass was studied in several experiments. Induction was found to be a chemical or hormonal differentiation that occurred as a response to both low temperature and short days. Little flowering resulted from day lengths above 12.5 hours, with 14 hours the upper limit. Induction occurred naturally during the fall. In 1949 and 1950, plants of local strains were induced to flower by November 1 at Ames, Iowa.

Induction of orchard grass and some other introduced grasses indigenous to the north temperature zone did not occur normally under spring conditions with prevailing cool temperature but long days. Other grasses of similar origin, such as smooth brome grass, flowered sparsely, while others such

as timothy flowered heavily. Many native grasses and those indigenous to southern latitudes were independent of the short-day and low temperature requirement. Orchard grass and Kentucky bluegrass could be made to flower by transferring to a cold greenhouse before April 1 and March 3 respectively.

Floral induction occurred when short days and low temperature were applied separately, *i.e.*, all short days were at a warm temperature, if the short days preceded the low temperature. Induction stimulus was found to be local, and axillary shoots developing after removal from induction conditions did not flower.

Long days and moderate temperature were necessary for initiation of floral primordia. Adverse short days for a 45-day period arrested development but did not destroy induction. Further floral development, the production of macroscopic flowers and fruits, was favored by long days and high nitrogen fertility.

The writers wish to express their appreciation to Dr. I. J. Johnson for helpful suggestions during the course of this study.

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ON THE MECHANISM OF ACTION OF GROWTH REGULATORS

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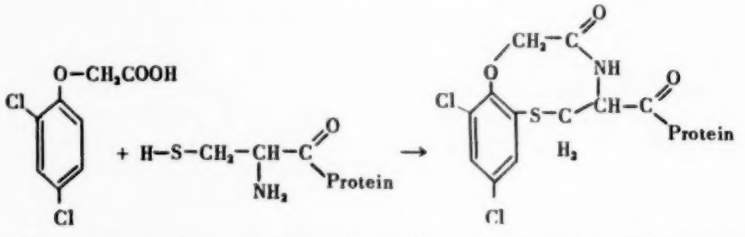
Introduction

Investigations of the effects of various analogues of indoleacetic acid, phenoxyacetic acid, phenylacetic acid and benzoic acid on the elongation of sections of Avena coleoptiles (**7, 8, 16, 17**) have led to the conclusion that plant growth-regulators react by a nucleophilic substitution at a position on the ring ortho to the carboxyl group or the side chain carrying this group and with the carboxyl group itself. The evidence at hand indicates that a cysteinyl unit of a protein is the most likely substrate for the two-point ortho reaction as illustrated below with 2,4-dichlorophenoxyacetic acid.



There are nine important experimental requirements, in addition to the requirement of an unsaturated ring and a carboxyl group first postulated by KOEPFLI *et al.* (**11**), which must be explained by any hypothesis which would correlate chemical structure of the growth regulators with physiological activity. These are (a) the effect of optical isomerism of the side chain (**18, 35, 36**), (b) the effect of geometrical isomerism of the side chain (**31, 32, 33**), (c) the ortho effect in benzoic acids (**1, 16**), (d) the ortho effect in phenoxyacetic acids (**7, 13, 17**), (e) the inhibiting effects of lactones (**6, 32**) and maleic hydrazide (**4, 9, 14, 40**), (f) the inhibiting effect of cysteine (**2**), (g) the inhibiting effects of reagents specific for sulfhydryl groups (**28, 29**), (h) the inactivity of phenoxyacetic acids without two open para positions (**13**), (i) the effect of electron-releasing and electron-attracting ring substituents (**16**). It is the purpose of this paper to present data on the relative activity of some growth regulators and to consider recent proposals for the reaction mechanism of the growth regulators in relation to the requirements listed above.

Methods

Seedlings of Avena plants grown in sand in darkness for 88 to 90 hours under conditions of constant temperature (24 ± 1° C) and humidity (85%)

were used as test material. The apical two millimeters of the coleoptile were removed and after two hours a single section, 3 ± 0.02 mm. in length, was cut from the apex with the primary leaf included within the coleoptile section. Fifteen such sections were floated on the surface of 25 ml. of a solution of the chemical in distilled water in a Petri dish. The compounds were tested over a concentration range of 10^{-5} to 10^{-4} M, and in some cases the higher concentrations included 5×10^{-4} M. When the concentration required to induce the growth response was great enough to give a pH of 5.4 or less, NaOH was added to raise the pH to 5.6 to 6.0. After 24 hours in the covered Petri dish in the dark room, the sections were removed and their length was measured with an ocular micrometer mounted in a binocular dissecting microscope at a magnification of 15 diameters.

Results and discussion

In formulating a measurement of the relative activity of chemical compounds in effecting the elongation of coleoptile sections several possibilities have been examined. The index of activity adopted here is the molar concentration of the compound causing a growth response compared with the molar concentration of indoleacetic acid (IAA) inducing the same growth response. An average elongation of the sections which is greater by 0.15 mm. (5% of the original length) than the elongation occurring in distilled water was made the standard of growth response. The concentration of IAA giving this growth response in the tests reported here is 5×10^{-5} M.

The relative activities of 117 compounds are listed in tables I and II. The only compound which has been found to have a greater activity than IAA is the 4-chloro-derivative, and the only compound with equal activity is α-naphthaleneacetonitrile. By the activity index used here α-naphthaleneacetic acid and 2,3,5-triiodobenzoic acid have activities which are one half that of IAA, and 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid have only one fourth the activity of IAA.

Following the report of Jones *et al.* (**10**) that indoleacetonitrile is active in causing the elongation of Avena coleoptile sections a number of nitriles were tested. As shown in table II α-naphthaleneacetonitrile is as active as IAA and more active than naphthaleneacetic acid, and phenylacetonitrile is more active than phenylacetic acid. Benzonitrile is inactive as are α-naphthonitrile and γ-phenoxybutyronitrile. Zimmerman and Wilcoxon (**39**) found α-naphthaleneacetonitrile caused an epinastic response which was slower than the response induced by other regulators and suggested the nitrile is hydrolyzed in the plant tissue to the acid. Veldstra (**30**) found both indoleacetonitrile and naphthaleneacetonitrile to be inactive in the pea test but active in root formation in cuttings and considered the latter activity to be due to hydrolysis. Since the only nitriles found to be active in the present investigation are those which may be hydrolyzed to give an acid which is active, it might be supposed that hydrolysis does take place in the tissue of the coleoptile, yet the same results would be obtained if the nitrile

TABLE I

RELATIVE ACTIVITY OF INDOLEACETIC ACID, PHENOXYACETIC ACID, AND DERIVATIVES IN PROMOTING GROWTH (ELONGATION) OF PLANT CELLS.

$$\text{Activity index} = \frac{\text{Molar conc. of 3-indoleacetic acid inducing an elongation of 0.15 mm.}}{\text{Molar conc. of growth regulator inducing an elongation of 0.15 mm.}} \times 100.$$

Compound	Activity Index
3-Indoleacetic acid (5×10^{-4} M)	100.0
4-Chloro-3-indoleacetic acid	140.0
2-Methyl-3-indoleacetic acid	1.5
4,7-Dichloro-2-methyl-3-indoleacetic acid	0.1
5,7-Dichloro-2-methyl-3-indoleacetic acid	1.5
3-Indolebutyric acid	15.0
3-Indolepropionic acid	1.5
Phenoxyacetic acid	0.03
2-Acetyl-5-methylphenoxyacetic acid	Inactive
o-Bromophenoxyacetic acid	0.1
m-Bromophenoxyacetic acid	2.5
p-Bromophenoxyacetic acid	1.5
2,4-Dibromophenoxyacetic acid	12.5
2,6-Dibromophenoxyacetic acid	Inactive
2,4,6,-Tribromophenoxyacetic acid	Inactive
o-Chlorophenoxyacetic acid	0.06
m-Chlorophenoxyacetic acid	2.0
p-Chlorophenoxyacetic acid	5.0
2,4-Dichlorophenoxyacetic acid	25.0
2,6-Dichlorophenoxyacetic acid	Inactive
3,5-Dichlorophenoxyacetic acid	Inactive
2,4,5-Trichlorophenoxyacetic acid	25.0
2,4,6-Trichlorophenoxyacetic acid	Inactive
2,4-Dichloro-6-methylphenoxyacetic acid	Inactive
2,4-Dichloro-5-nitrophenoxyacetic acid	0.2
2-Ethyl-4-chlorophenoxyacetic acid	Inactive
o-Iodophenoxyacetic acid	0.1
p-Iodophenoxyacetic acid	Inactive
2,4-Diiodophenoxyacetic acid	Inactive
o-Isopropylphenoxyacetic acid	Inactive
2-Isopropyl-4-chloro-5-methylphenoxyacetic acid	Inactive
o-Methoxyphenoxyacetic acid	Inactive
m-Methoxyphenoxyacetic acid	0.1
p-Methoxyphenoxyacetic acid	0.03
o-Methylphenoxyacetic acid	0.2
m-Methylphenoxyacetic acid	0.07
p-Methylphenoxyacetic acid	0.05
2,4-Dimethylphenoxyacetic acid	0.5
2,5-Dimethylphenoxyacetic acid	0.2
3,5-Dimethylphenoxyacetic acid	Inactive
2,4,6-Trimethylphenoxyacetic acid	Inactive
o-Nitrophenoxyacetic acid	Inactive
m-Nitrophenoxyacetic acid	0.2
p-Nitrophenoxyacetic acid	0.1
2,4-Dinitrophenoxyacetic acid	Inactive

TABLE II

RELATIVE ACTIVITY OF SOME REGULATORS IN PROMOTING GROWTH (ELONGATION) OF PLANT CELLS.

$$\text{Activity index} = \frac{\text{Molar conc. of 3-indoleacetic acid inducing an elongation of 0.15 mm.}}{\text{Molar conc. of growth regulator inducing an elongation of 0.15 mm.}} \times 100.$$

Compound	Activity index	Compound	Activity index
Benzoic acid	Inactive	*o*-Fluorobenzoic acid	Inactive
o-Aminobenzoic acid	Inactive	*m*-Fluorobenzoic acid	Inactive
m-Aminobenzoic acid	Inactive	*p*-Fluorobenzoic acid	Inactive
2-Amino-3,5-diiodobenzoic acid	Inactive	*o*-Iodobenzoic acid	Inactive
o-Bromobenzoic acid	0.1	*m*-Iodobenzoic acid	Inactive
m-Bromobenzoic acid	Inactive	*p*-Iodobenzoic acid	Inactive
p-Bromobenzoic acid	Inactive	2,3,5-Triiodobenzoic acid	50.0
o-Chlorobenzoic acid	0.05	3,4,5-Triiodobenzoic acid	Inactive
m-Chlorobenzoic acid	Inactive	2,4,6-Triisopropylbenzoic acid	Inactive
p-Chlorobenzoic acid	Inactive	*o*-Methylbenzoic acid	Inactive
2-Chloro-5-nitrobenzoic acid	Inactive	*m*-Methylbenzoic acid	Inactive
2,4-Dichlorobenzoic acid	Inactive	*p*-Methylbenzoic acid	Inactive
2,5-Dichlorobenzoic acid	1.0	2,6-Dimethylbenzoic acid	0.05
2,6-Dichlorobenzoic acid	0.1	2,4,6-Trimethylbenzoic acid	Inactive
Pentachlorobenzoic acid	Inactive	2,6-Dimethyl-4-methoxybenzoic	Inactive
2,4,6-Triethylbenzoic acid	Inactive	2,6-Dimethoxybenzoic acid	Inactive
m-Ethoxybenzoic acid	Inactive	2-Methoxy-3,5-dichloro-4,6,-dimethylbenzoic acid	Inactive
p-Ethoxybenzoic acid	Inactive		
o-Hydroxybenzoic acid	Inactive	*o*-Nitrobenzoic acid	0.1
m-Hydroxybenzoic acid	Inactive	*m*-Nitrobenzoic acid	Inactive
p-Hydroxybenzoic acid	Inactive	*p*-Nitrobenzoic acid	Inactive
		3,5-Dinitrobenzoic acid	Inactive
Phenylacetic acid	1.0	Benzonitrile	Inactive
α-Aminophenylacetic acid	Inactive	Benzothiazyl-2-oxyacetic acid	0.5
p-Aminophenylacetic acid	0.05	α-Naphthaleneacetic acid	50.0
m-Fluorophenylacetic acid	1.5	α-Naphthaleneacetonitrile	100.0
p-Fluorophenylacetic acid	1.5	α-Naphthonitrile	Inactive
2,5-Dihydroxyphenylacetic acid	0.02	β-Naphthoxyacetic acid	0.8
p-Iodophenylacetic acid	Inactive	2-Phenanthreneacetic acid	Inactive
2,5-Dimethoxyphenylacetic acid	Inactive	3-Phenanthreneacetic acid	Inactive
2,4-Dimethylphenylacetic acid	0.5	γ-Phenoxybutyronitrile	Inactive
3,5-Dimethylphenylacetic acid	0.5	α-Phenoxypropionic acid	0.5
2,4,6-Trimethylphenylacetic acid	Inactive	Phenylacetonitrile	2.0
		γ-Phenylbutyric acid	1.5
p-Nitrophenylacetic acid	Inactive	2,4,6-Trimethylphenylbutyric acid	Inactive
2,4-Dinitrophenylacetic acid	Inactive		
p-Phenylphenylacetic acid	Inactive	*p*-Chlorophenylglycine	1.0
Diphenylacetic acid	Inactive	β-Phenylpropionic acid	Inactive

group reacts in similar fashion to the carboxyl group in the two-point reaction. Further investigation of this problem is planned.

One proposal concerning the chemical structure required to effect cell elongation is that of WAIN (**36**) which emphasizes the presence of a hydrogen atom in the alpha position of the aryloxyacetic acids and suggests that the growth regulator which effects cell elongation is adsorbed onto an active

site by three points: the carboxyl group, the unsaturated ring, and an alpha hydrogen. Certain observations are not in accord with this hypothesis however. Phenoxyacetic acid according to the hypothesis should be more active than α-methylphenoxyacetic acid which is very active, yet as shown in table I phenoxyacetic acid has very low activity and many investigators have reported it to be completely inactive. The relative activities of seven benzoic acids which cause cell elongation although they do not have an alpha hydrogen atom are given in table II. WAIN (**36**) believes the benzoic acids do not have the same mechanism of reaction as other growth regulators, but ZIMMERMAN and HITCHCOCK (**38**) have found that 2,3,6-trichlorobenzoic acid brings about the same responses of epinasty, proliferation of tissue, formation of adventitious roots, modification of leaves, and development of parthenocarpic fruit that are effected by other regulators. In this laboratory 2,3,5-triiodobenzoic acid has been found to cause parthenocarpic fruit to develop when applied to ovaries of tomato in concentrations of 0.3 and 0.03% in lanolin paste (**22**). The compound was also found to cause greater development of adventitious roots on cuttings of Euonymus at 10^{-6} M and 10^{-7} M. SNYDER has reported similar effects of the compound on the rooting of cuttings of Coleus when applied at a concentration of 1 p.p.m. (**25**).

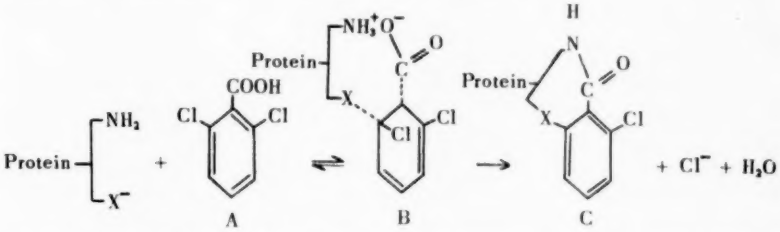
In active compounds of the type of cis cinnamic acid, the alpha hydrogen atom, the benzene ring, and the carboxyl group do not have the same spatial relation as in the saturated side chains of phenoxyacetic acids or phenylacetic acids and adsorption at precisely the same three points would therefore not be possible. It also seems unlikely that the active compounds, γ-phenylbutyric acid and 3-indolebutyric acid (table II), could be held by the carboxyl group and an alpha hydrogen atom and still have the ring adsorbed at the same point which holds the ring of phenylacetic acid or phenoxyacetic acid which have much shorter side chains.

The effect of alpha substitutions in the phenoxyacetic acids can be explained by the steric hindrance of the carboxyl group by the alpha alkyl groups. It is a well known fact (**5, 20**) in organic chemistry that the introduction of substituents on the alpha carbon atom greatly slows down the rate of reaction of the functional group, in this case the carboxyl group. This general problem has been discussed by WHELAND (**37**). Thus, if the carboxyl group of a growth regulator reacts chemically then it is quite reasonable to expect that two groups on the alpha carbon atom would slow this reaction to a point where the activity of the compound is negligible. This possibility is even more likely in the case of a biological reaction brought about by large enzymatic catalysts which in themselves would require considerable freedom of approach to the reaction site than it would be with simple organic molecules.

Although KÖGL and VERKAAIK (**12**) found that the D and L isomers of α-methylindoleacetic acid are of equal activity, WAIN (**36**) and THIMANN (**26**) have reported that in a number of phenoxyacetic acids such as 2,4-dichloro-α-methylphenoxyacetic acid with one alkyl group on the alpha car-

bon, one optical isomer is active while the other is not. According to Wain's hypothesis one optical isomer would fit the three points of adsorption while its mirror image would not and a compound with both hydrogens substituted by alkyl groups would also not fit. However, the difference in activity of optical isomers resulting from an asymmetric carbon atom may also be explained by differences in reaction rates resulting from interference in combining with another asymmetrical center. As an example of this interference, it has been shown (**5**) that the D form of mandelic acid reacts much faster than the L form with L-menthol in an esterification reaction. The demonstration by WAIN and coworkers (**24, 36**) of the antagonism of the inactive di-alpha substituted phenoxyacetic acids toward the effect of such regulators as 2,4-dichlorophenoxyacetic acid and the antagonism of the inactive isomer of 2,4,5-trichlorophenoxypropionic acid toward the effect of the active isomer may be explained also on the basis of a two-point ortho reaction mechanism involving the carboxyl group. Despite the one or two alpha substituents the carboxyl group would be able to form a salt linkage with the basic group of the protein thus hindering the regulator from reacting at this point. Although a salt linkage could be formed the subsequent amidization would be prevented by the methyl groups. Compounds with steric hindrance of the carboxyl group but with open ortho positions could react at the ortho positions and prevent the regulator from completing the two-point attachment.

At first glance one might expect the di-ortho substituted benzoic acids to contain a sterically hindered carboxyl group as in the di-alpha substituted phenoxyacetic acids. Such benzoic acids are very slow to give many normal reactions (**37**), but the mechanism postulated for 2,6-dichloro- and 2,3,6-trichlorobenzoic acids (**16**) involves two reactions, one of which is a nucleophilic substitution on the aromatic ring as follows:



To understand the steric significance of the intermediate, B, for the amidization of A and C, one must consider the structure of B in detail. The available chemical and theoretical evidence (**3**) shows that in nucleophilic substitution reactions an intermediate complex of type B is formed. Many of these complexes have considerable stability and some may even be isolated as such. When such a substitution occurs the usual aromatic resonance of the ring is momentarily destroyed and thus the carbon atoms 1 and 6 may assume the tetrahedral structure. In the complex B above, carbon

atoms 1 and 6 may now possess the usual tetrahedral configuration with the carboxyl group out of the plane of the ring. The first stage of the reaction is salt formation of the carboxyl group with an amine group, then nucleophilic displacement of the ortho chlorine atom. In the activated complex state, the carboxyl group will be out of the plane of the ring and on the same side of the ring from which the nucleophilic group, X, is attacking the chlorine atom. The important point to note is that in this position the carboxyl group is attached to a ring carbon which in turn is attached to only two other atoms, *i.e.*, two ring carbon atoms, which means that the carboxyl group will be relatively unhindered sterically for the amidization reaction which may now be promoted enzymatically. Thus, sterically, the structure of B would be analogous to a mono-alpha substituted phenoxyacetic acid, except that the latter would carry a hydrogen atom on the alpha carbon while the former would carry a momentarily unshared pair of electrons which might be satisfied by a proton or an enzyme.

All of the benzoic acids listed in table II which cause cell elongation have a halogen atom or nitro group in the ortho position except 2,6-dimethylbenzoic acid. The importance of the halogen atom at the ortho position in determining the activity of the benzoic acids is demonstrated by the activity of 2,3,5-triiodobenzoic acid compared with the lack of activity of 2-amino-3,5-diiodobenzoic acid and 3,4,5-triiodobenzoic acid.

Although it was first shown by KOEPFLI *et al.* (**11**) that cis cinnamic acid causes cell elongation and the trans form does not, the importance of the geometrical isomerism of the side chain in determining the activity of all growth regulators with such forms has been emphasized by VELDSTRA and coworkers (**31, 32, 33, 34**). The structure of the cis isomers is such that the side chain with the carboxyl group is held out of the plane of the ring because of steric hindrance. Veldstra has proposed that the active compound must have a non-polar part (ring system) with an acidic polar group (preferably a carboxyl group) situated out of the plane of the ring as far as possible or as frequently as possible.

The mechanism of action of growth regulators as interpreted by Veldstra is inadequate in several respects. In the first place, as THIMANN (**26**) has pointed out, if having the carboxyl group out of the plane of the ring is the principal requirement, then the most active compounds should be those such as cis cinnamic acid in which this position is only one of many positions attainable by free rotation, however such compounds are not the most active. Even more difficult to explain on the basis of Veldstra's hypothesis is the inactivity of certain benzoic acids. VELDSTRA (**31**) has shown spectrophotometrically that in 2,6-dichlorobenzoic acid and 2,3,6-trichlorobenzoic acid the ortho substituents force the carboxyl group into a position at right angles to the benzene ring and the activity of these compounds is predicted by his hypothesis. Benzoic acids with substituents in both ortho positions are not necessarily active, however (**16**). Included in table II are several benzoic acids which have groups in both ortho positions and which are inactive.

These acids are 2,6-dimethoxybenzoic acid, 2,4,6-trimethylbenzoic acid, 2,4,6-triethylbenzoic acid, 2,4,6-triisopropylbenzoic acid, 2,6-dimethyl-4-methoxybenzoic acid and 2-methoxy-3,5-dichloro-4,6-dimethylbenzoic acid. Certainly in these molecules there is no reason to doubt that the favored position of the carboxyl group is out of the plane of the ring and their lack of activity is not explained by Veldstra's hypothesis. It should be noted here that 2,6-dimethylbenzoic acid is weakly active. The supposition that the hydrophilic/lipophilic character of the molecule effects its activity does not help in understanding the effect of ring substituents in benzoic acid. For example, in comparing the very active 2,3,6-trichlorobenzoic acid with the completely inactive 2,4,6-trimethylbenzoic acid it seems highly unlikely that these two molecules would differ greatly in their hydrophilic/lipophilic ratio. Not only is the atomic volume of these groups the same (**23**), but they are both also weakly polar, fat soluble groups. In both molecules the chlorine atoms and methyl groups in the ortho positions would hold the carboxyl group in precisely the same position with respect to the ring.

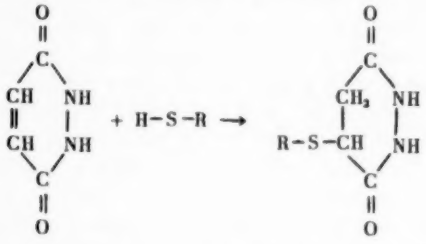
The variation in activity shown by the phenylacetic acid and phenoxyacetic acid series is also not explained by Veldstra's hypothesis. In tables I and II are found several derivatives of these acids in which both ortho positions are substituted, thus preferentially holding the side chain out of the plane of the ring, yet all of them are inactive. These compounds include the 2,6-dichloro-, 2,6-dibromo-, 2,4,6-trichloro-, 2,4,6-tribromo-, 2,4,6-trimethyl-, and the 2,4-dichloro-6-methyl- derivatives of phenoxyacetic acid and 2,4,6-trimethylphenylacetic acid. Also inactive is 2,4,6-trimethyl-γ-phenylbutyric acid.

Any hypothesis attempting to correlate chemical structure and physiological activity must consider the important effect of di-ortho substitution on the activity of benzoic acids, phenylacetic acids and phenoxyacetic acids. Slight differences in adsorption characteristics are not adequate to account for the effects on activity. The existence of the ortho effect is illustrated strikingly by a comparison of the completely inactive 2,4,6-trichlorophenoxyacetic acid with the active 2,4,5-trichlorophenoxyacetic acid (table I). Since these molecules are made up of exactly the same atoms their hydrophilic/lipophilic ratios would be the same, and the first compound which has the carboxyl group held out of the plane of the ring should, on the basis of Veldstra's hypothesis, be more active, yet it is inactive.

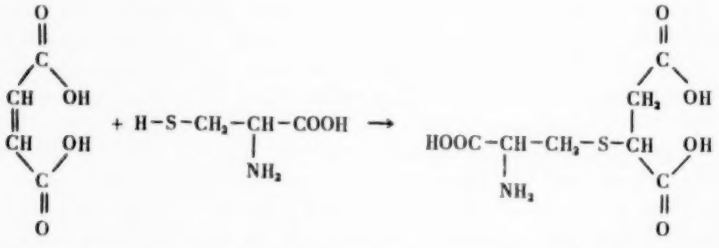
Veldstra and Booij (**33**) attempted to obtain a correlation between activity and the suppression of the polarigraphic oxygen maximum. They reasoned that the degree of adsorption of the organic compound to the mercury electrode (as measured by the suppression) would parallel its adsorption to a protoplasmic membrane. This ingenious experiment seemed to be a way to measure the hydrophilic/lipophilic coefficient, and it appeared in this first work that there was some correlation; however, a more extensive investigation (**19**) using his polarigraphic technique shows no correlation between activity and adsorption on the mercury electrode. From this in-

vestigation and from a general consideration of the great difference in activity of very similar compounds such as those mentioned above, it would seem that knowledge of the hydrophilic/lipophilic ratio would be of little help in predicting or explaining relative activity except in extreme cases.

Neither the hypothesis of Veldstra nor that of Wain takes sufficient account of the effects of the type or position of the groups attached to the benzene ring. Neither view explains the effects of the compounds which are inhibitors of growth. The effects of lactones, cysteine, and reagents specific for the sulfhydryl group have been discussed in an earlier paper (**8**). It seems likely that the growth inhibitor, maleic hydrazide, reacts in the same fashion as the lactones by reaction with a sulfhydryl group as follows:



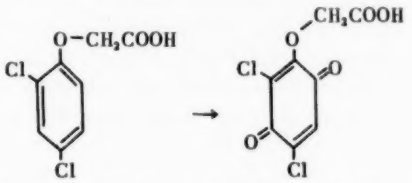
There are many instances of reaction between sulfhydryl groups and activated double bonds. MORGAN and FRIEDMANN (**15**) have shown that maleic acid will react with a variety of thiol compounds and that cysteine reacts in 93% yield as follows:



Undoubtedly a study of other stable organic compounds with double bonds activated by groups such as amides, esters, ketones, and nitriles would lead to the discovery of many compounds quite toxic to plant cells.

Another recent study of the structure and activity of growth regulators is that of LEAPER and BISHOP (**13**). They have prepared and tested all of the possible mono-, di-, and trichlorophenoxyacetic acids. Their tests indicated that unless the compound contained an open ortho position and in addition an open position para to the ortho position, the molecule was inactive or of very low activity. Thus they found that 3,5-dichlorophenoxyacetic acid was completely inactive. That this compound is inactive has been confirmed by THIMANN (**27**) using the pea test, and in the investigations re-

ported here it was found to be inactive in the Avena section test. Leaper and Bishop have made the reasonable suggestion that the necessity for the two open para positions is explained by the oxidation of the growth regulator to quinoid compounds in plant cells as follows:



To test this hypothesis 2,5-dihydroxyphenylacetic acid was synthesized and tested for physiological activity. As shown in table II, this compound has lower activity than phenylacetic acid. Since the 2,5-dihydroxy- compound should readily form a quinone, the oxidation to quinoid compounds does not appear to be involved in the mechanism of action of phenylacetic acids. Additional evidence against the quinone hypothesis is found in the work of THIMANN (**26**) in which methylbenzoquinonethioacetic acid, trimethylbenzoquinonethioacetic acid and 1,4-naphthoquinone-2-butyric acid were found to be inactive. Another objection to the quinone hypothesis is raised by the fact that 4-chloroindoleacetic acid is more active than indoleacetic acid as shown in table I. Unless one postulates the displacement of the halogen in this compound, para quinone formation could result only through the destruction of the five-membered ring with the quinone forming through the 5 position and the point at which the nitrogen was attached. The quinone hypothesis fails to explain the activity of 2-methyl-5,7-dichloro-3-indoleacetic acid which has all positions blocked against quinone formation. Also α-naphthaleneacetic acid could not form a para quinone unless both rings are involved. Thus at present it is unlikely that quinone formation explains the requirement of an open set of para positions in the phenoxyacetic acids.

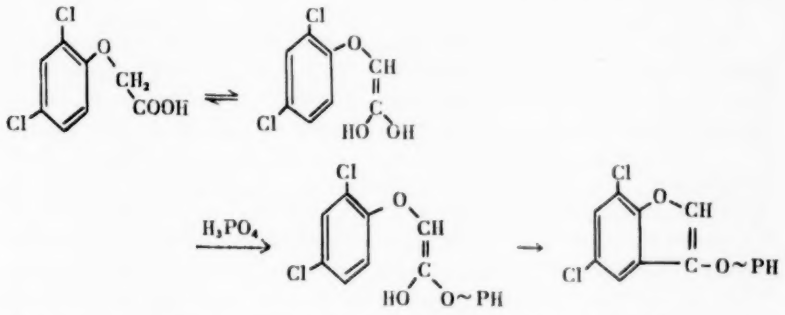
THIMANN (**26, 27**) has advanced some suggestions for the correlation of structure and activity of plant growth-regulators. He accepts the view that the regulators react chemically with a substrate and states that the reaction occurs at either the 2, 4, or 6 position on the ring of phenylacetic acids and phenoxyacetic acids with the nucleophilic influence divided between these three positions, with the result that no one of them is highly active. Thimann is led to the difficult position of this postulate by the observation that phenoxyacetic acid is inactive and the introduction of one halogen atom in the 2, 4, or 6 position increases the activity while the introduction of two halogen atoms as in 2,4-dichlorophenoxyacetic acid increases activity to a maximum. Thus the occupation of one or more of the ortho or para positions is believed to lead to intensification of the nucleophilic influence at the remaining positions. Not only does all theoretical and experimental work in organic chemistry point against such a hypothesis, but close examination of the structure of active compounds offers little support for it.

THIMANN (**27**) has found by means of the pea test that 2,6-dichlorophenoxyacetic acid is slightly active. A sample of this compound, kindly supplied by Dr. Thimann, was found to be inactive in the Avena section test in this investigation. LEAPER and BISHOP (**13**) also report the compound to be completely inactive. At most, 2,6-dichlorophenoxyacetic acid must be regarded as of doubtful activity, although on the basis of Thimann's hypothesis one would expect it to be highly active. Thimann reports also that 2,4,6-trichloro- and 2,4,6-tribromophenoxyacetic acids are slightly active, although all other investigators have found these compounds to be inactive. The suggestion that the activity found may be due to hydrolysis seems unlikely for two reasons. First, it has been recognized for some time that an —OH group decreases rather than increases activity (**27**). Secondly, even though a chlorine atom were replaced by a less effective hydroxyl group, the position would still be blocked for chemical reaction by the —OH group.

Thimann states that activation is by electron release to the 2, 4, and 6 positions and thus alkyl groups and particularly methyl groups should be effective in the activation of the phenoxyacetic acids and phenylacetic acids. Considerable evidence exists to show that such activation does not occur. The best procedure is to introduce the group in the acids without any other complicating groups attached to the ring. As is shown in table I, *o*-methyl-, *p*-methyl-, *m*-methyl-, 3,5-dimethyl-, 2,4,6-trimethyl-, *o*-ethyl-, and *o*-isopropylphenoxyacetic acids are completely inactive or of low activity. On the basis of Thimann's hypothesis it is quite surprising that 2,4-dimethylphenoxyacetic acid has a very low activity since it is analogous to the very active 2,4-dichlorophenoxyacetic acid. The small activity of the *p*-methyl compound is also noteworthy. Just as interesting is the case of 2,4-dimethylphenylacetic acid which is shown in table II to be one half as active as phenylacetic acid. Here the methyl groups actually lower the activity. These examples are compounds substituted with one type of group; with substitution of sufficiently strong electron-attracting groups of other types the effect could be reversed. So far the only groups which have been found to confer activity individually on the phenoxyacetic acid ring are the halogens and the nitro group.

Thimann points out that resonance in the phenoxyacetic acids increases the electron density at the 2, 4, and 6 positions thus accounting for the activity at these points. The increased electron density is supposed to indicate reactivity with a weakly charged positive substrate which does not seem unreasonable for the phenoxyacetic acid series. With the halogenated benzoic acids however a weakly charged positive substrate would be incapable of successful attack and substitution on the electron-deficient ring. Moreover, according to the hypothesis of Thimann, substitution of the ring of benzoic acid with electron-releasing groups such as —OH, —OR, and —NH_2 should be more effective than the introduction of electronegative halogen atoms, yet, as shown in table II, such substituted benzoic acids are completely inactive.

RHODES and ASHWORTH (**21**) have suggested that the phenylacetic and phenoxyacetic acids may be involved in a cyclization reaction after enolization as follows:



This type of reaction would explain Wain's suggestion that a hydrogen atom is necessary on the alpha carbon atom of the side chain. However, this hypothesis is subject to the same limitations discussed in connection with the work of Wain and Osborne.

Although the ortho reaction mechanism seems to be the best working hypothesis for correlating the present data, it is not perfect. It does not offer an obvious explanation for the lack of activity of 2,4-dichlorobenzoic acid compared with the activity of 2,5-dichlorobenzoic acid nor for the activity of 2,6-dimethylbenzoic acid. A more difficult point to explain is the activating effect of a methyl group on the alpha carbon atom as shown by the greater activity of α-methylphenoxyacetic acid compared with phenoxyacetic acid which is weakly active. Also at the present time it is not clear why 3,5-dichlorophenoxyacetic acid is an inactive compound.

Summary

The relative activities of 117 chemical compounds including derivatives of indoleacetic acid, phenoxyacetic acid, phenylacetic acid and benzoic acid have been determined on the basis of the molar concentration required to induce a minimal response in elongation compared with the molar concentration of indoleacetic acid inducing the same elongation. With such a measurement of relative activity only 4-chloro-3-indoleacetic acid is more active than indoleacetic acid (IAA). The same degree of activity as IAA is shown only by α-naphthaleneacetonitrile. The relative activity of naphhaleneacetic acid and 2,3,5-triiodobenzoic acid is half that of IAA, and 2,4-dichlorophenoxyacetic acid and 2,4,5-trichlorophenoxyacetic acid have one fourth the activity of IAA.

Recent hypotheses concerning the correlation of chemical structure with physiological activity of plant growth regulators have been examined as explanations for the activity or lack of activity of di-ortho alkyl substituted

analogues and the low activity or inactivity of mono-alkyl substituted analogues. It is concluded that the two-point ortho reaction mechanism affords the best working hypothesis although a few compounds at present do not seem to fit the hypothesis.

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PYRIDINE NUCLEOTIDE–NITRATE REDUCTASE FROM EXTRACTS OF HIGHER PLANTS[1]

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Introduction

The major source of nitrogen for most higher plants and many microorganisms is nitrate, yet natural mechanisms by which it is reduced have remained obscure. Most of the experimental evidence concerning assimilation of nitrate by higher plants has been included in reviews by NIGHTINGALE (**24, 25**), STREET (**29**), and VIRTANEN and RAUTANEN (**30**). The early work of ECKERSON (**9**) showed that nitrite was formed when the expressed sap of plants was incubated with nitrate and glucose. Since long incubation periods were used in these experiments, the results do not show conclusively whether the nitrate reduction was catalyzed by enzymes from the sap or from contaminating microörganisms. Experiments by various investigators (**9, 12**) have demonstrated the formation of nitrite in tissues of nitrogen-deficient plants after nitrate had been added to nutrient solutions and had accumulated in various organs. Nitrite appearance was usually associated with the disappearance of carbohydrates and an increase in respiration. ANDERSON (**1**) reported the detection of nitrite in leaves and shoots of 25 plant species, and stated that the agent responsible for its formation was thermolabile and oxidizable. BÜRSTROM (**6**), ECKERSON (**10**), and others have shown that light is involved in nitrate assimilation by the aerial portions of plants. The mechanism of this effect, however, has not been apparent. In a recent study, BHAGVAT (**29**) reported that the aldehyde oxidase system, which catalyzes the oxidation of aldehyde by oxygen or by nitrate in absence of oxygen, is present in the potato. The physiological importance of this enzyme in nitrate assimilation has not been determined.

It is generally concluded (**24, 25, 29, 30**) that nitrite is a probable intermediate in nitrate metabolism and that the process of nitrate reduction is enzymatic. The characteristics and requirements of a widely distributed enzyme system in higher plants likely to play an important role in nitrate reduction have not been established. It is the purpose of this paper to describe the purification and properties of a pyridine nucleotide–nitrate reductase from soybean leaves already reported from this laboratory (**11**), and to present evidence for its occurrence in other higher plant species.

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Experimental methods and materials

Source of enzyme

The crude enzyme extract used in the purification procedure was obtained from the primary leaves of 8- to 10-day-old soybean seedlings (*Glycine max* Merr., variety Odgen) grown in flats (two feet by two feet) filled with sand or vermiculite. Each flat was supplied each day with approximately two liters of nutrient solution containing 0.005 M $Ca(NO_3)_2$, 0.0005 M KH_2PO_4, 0.002 M $MgSO_4$, 0.0025 M K_2SO_4, 0.5 p.p.m. Fe as $FeSO_4$, 0.02 p.p.m. Cu as $CuSO_4$, 0.015 p.p.m. Mo as Na_2MoO_4, and 0.25 p.p.m. B, Mn, and Zn as H_3BO_3, $MnSO_4$, and $ZnSO_4$, respectively. Solutions were prepared with demineralized water and adjusted to pH 5.8. Flats also were supplied daily with a volume of demineralized water equal to that of the nutrient solution.

The crude extract (fraction I) was made by grinding one weight of fresh primary leaves, three weights of cold 0.1 M K_2HPO_4 buffer (pH 9.0), and two weights of alumina powder (Alcoa A-301) in a Waring Blendor for two minutes at 4° C. The mixture was further ground for three minutes in a Ten Brock homogenizer at 0 to 4° C, then centrifuged in a Servall centrifuge at 20,000 times gravity for 10 minutes at 4° C. The supernatant fluid which was green in color but clear was used for purification. Measurement of activities in homogenates and crude extracts indicated that approximately 10% of the enzyme was dissolved by this procedure. Homogenates used in nutrition and survey experiments were prepared by grinding one weight of fresh tissue with three weights of cold 0.1 M phosphate buffer at pH 7.0, in a cold mortar, then in a Ten Brock homogenizer at 0 to 4° C.

The effect of nitrogen source on the enzyme activity was studied with soybean plants grown for five days (after a four-day germination period) in two-liter beakers containing nutrient solutions as follows: (a) nitrate solution, as indicated above for seedlings, (b) ammonium solution, containing nutrients indicated above, with the exception that 0.005 M $(NH_4)_2SO_4$ was substituted for $Ca(NO_3)_2$, (c) solution without nitrogen containing nutrients indicated above, with the exception that 0.005 M $CaCl_2$ was substituted for $Ca(NO_3)_2$. These solutions were diluted with an equal volume of water and adjusted to pH 5.8. The various plant species used in the survey were grown in six-inch ceramic pots, filled with vermiculite, and were supplied with nutrient solution and water as indicated for soybean seedlings grown in flats. Deficiencies of Fe, Mn, B, Zn, Mo, and N were obtained in 35-day-old soybean plants by a method previously described (**21**).

Co-factors, substrates and other materials

Triphosphopyridine nucleotide (TPN) of approximately 75% purity was prepared from sheep liver by a procedure utilizing ion exchange chromatography (unpublished method of Dr. A. Kornberg and Dr. B. L. Horecker). The reduced derivative of triphosphopyridine nucleotide (TPNH) was prepared enzymatically as follows: 40 mg. of TPN were dissolved in 15.4 ml.

of 0.1 M phosphate buffer, at pH 7.5, and 0.8 ml. of 0.1 M $MgCl_2$, and 3 ml. of 0.05 M D-isocitrate were added. The reaction was started by addition of 0.8 ml. of a phosphate buffer (pH 7.5) extract of washed, acetone-dried, pig heart, containing isocitric dehydrogenase (**26**). The reaction was allowed to proceed at room temperature for 30 to 45 minutes, and at intervals during this period 0.05 ml. aliquots were removed, diluted to 3.0 ml. with 0.1 M phosphate buffer at pH 7.5, and optical densities determined in a Beckman spectrophotometer at 340 mμ. The reaction was considered complete when a maximum increase in optical density was obtained. At the end of the reaction, the TPNH preparation was adjusted to pH 9.0 to 9.5, placed in a boiling water bath for three minutes, centrifuged for five minutes and the supernatant solution was used as a source of TPNH. This solution was shown to be active enzymatically by preliminary tests with partially purified glutathione reductase from peas (**20**). With this system the absorption decreased to the original starting value. The concentration of TPNH was determined spectrophotometrically by use of the extinction coefficient of 6.24×10^6 sq. cm. $\times$ mole^{-1} at 340 mμ (**14**).

Diphosphopyridine nucleotide (DPN) of 65% purity was obtained from the Sigma Chemical Company, and the reduced derivative of diphosphopyridine nucleotide (DPNH) was prepared from this by a method described elsewhere (**27**). Flavin-adenine dinucleotide (FAD) of approximately 60% purity, prepared from yeast by the method of WARBURG and CHRISTIAN (**33**), was kindly furnished by Dr. L. A. Heppel. The FAD of 81% purity was obtained from the Sigma Chemical Company and the flavin mononucleotide (FMN) from Nutritional Biochemicals, Inc. In certain experiments as indicated, a boiled extract of acetone-dried pig heart was used as a source of FAD. The concentrations of FAD solutions were determined spectrophotometrically by the use of the extinction coefficient of 1.13×10^7 sq. cm. $\times$ moles^{-1} at 455 mμ (**33**).

Cytochrome c, used in the cytochrome c reductase assay (**15**), was obtained from the Sigma Chemical Company. The sodium salt of D-isocitric acid of 70% purity (30% citric acid) was prepared by ion exchange chromatography (**7**) from extracts of powdered Bryophyllum leaves kindly supplied by Dr. H. B. Vickery. The apoenzyme of D-amino acid oxidase was prepared from pig kidney, according to the method of NEGELEIN and BROMEL (**23**), modified by an unpublished procedure of Dr. Wilhelm Frisell. Purified phosphogluconic dehydrogenase and the barium salt of phosphogluconic acid were kindly furnished by Dr. B. L. Horecker. Snake venom, used as a source of nucleotide pyrophosphatase, was obtained from the Ross Allen Reptile Institute.

Grana were prepared from mature leaves of 18- to 21-day-old soybean plants by a modification of the method used by VISHNIAC and OCHOA (**31**). One weight of leaves and two weights of cold 0.05 M phosphate buffer (pH 7.0) containing 0.5% KCl were ground in a mortar at 0 to 4° C. The macerated material was squeezed through four layers of cheesecloth and

centrifuged at 2000 × gravity for 10 minutes at 0 to 4° C. The supernatant fluid, which was used as a source of grana, contained a small number of intact chloroplasts. Removal of soybean grana by centrifugation, followed by resuspension in the above buffer, resulted in almost complete loss of photochemical activity. The loss also was obtained when grana were prepared in 0.5 M sucrose, instead of phosphate buffer containing KCl. Chlorophyll content of grana was determined by the method of MacKinney (**19**).

Standard nitrate reductase assay

Enzyme activity was measured by colorimetric determination (**28**) of the nitrite formed during a 10-minute incubation period at 28° C. The procedure was as follows: 0.1 ml. of 0.1 M KNO_3 (10 μM), 0.11 ml. of 0.5 M pH 7.0 phosphate buffer (55 μM), 0.05 ml. of 2.6×10^{-5} M FAD (1.3×10^{-3} μM), and 0.04 ml. of 2×10^{-3} M TPNH (0.08 μM) were added to a small test tube, and the reaction was started by adding 0.2 ml., or quantities indicated, of enzyme extract. The final volume was 0.5 ml. The mixture was allowed to incubate for 10 minutes, then the reaction was stopped by adding 0.9 ml. of water and 0.5 ml. of sulfanilamide reagent, followed immediately with 0.5 ml. *N*-(1-naphthyl)-ethylenediamine hydrochloride solution. After 10 minutes, the optical density was determined with a Klett colorimeter using a 540 mμ filter. Optical densities of control reaction mixtures containing all constituents except TPNH were used to correct for turbidity. There was no chemical reduction of nitrate to nitrite by TPNH.

When homogenates or extracts high in protein were used as enzyme sources, 0.9 ml. of saturated $(NH_4)_2SO_4$ solution was added to each reaction mixture at the end of the incubation period, instead of 0.9 ml. of water. The sulfanilamide and *N*-(1-naphthyl)-ethylenediamine hydrochloride reagents were added, mixtures were centrifuged, and optical densities determined as above. The $(NH_4)_2SO_4$ precipitate adsorbed some of the diazo compound, and, therefore, the experimental error was increased when this procedure was used. Tests showed that no measurable quantity of nitrite disappeared in experiments utilizing enzyme extracts under the conditions described; however, there was some disappearance during long incubation periods using homogenates. This was reduced by including a final concentration of 10^{-3} M $NH_2OH \cdot HCl$.

One unit of the soybean nitrate reductase is defined as that amount of enzyme which will catalyze the formation of 10^{-3} micromoles of nitrite in five minutes when carried out under the conditions described, with the exception of time. In the experiments reported, units of enzyme were determined by incubating reaction mixtures for 10 minutes and dividing the $\mu M \times 10^{-3}$ formed by 2. This unit is equivalent to that of the Neurospora enzyme (**22**). Specific activity is defined as the units of activity per mg. of protein which was determined by the method of Lowry *et al.* (**17**). The enzyme activities of homogenates are expressed on a dry weight basis, as indicated.

Experimental results

Purification of enzyme

Crude extracts (fraction I) of the primary leaves of 8- to 10-day-old soybean seedlings were purified by a method involving calcium phosphate gel adsorption and ammonium sulphate precipitation. All steps of the purification were carried out at 0 to 4° C. The various fractions were centrifuged at a force of approximately 3000 times gravity. The pertinent data of a typical purification described in detail below are recorded in table I.

Ninety-five milliliters of calcium phosphate gel (11 mg. per ml.) were added to 190 ml. of crude extract obtained from 60 gm. of primary leaves. The suspension was allowed to stand 10 minutes in the cold, then was centrifuged for five minutes and the supernatant liquid was discarded. The gel, containing the adsorbed enzyme, was mixed with 95 ml. of 0.1 M phosphate buffer at pH 7.5, and allowed to stand for one minute. The gel suspension

TABLE I

PURIFICATION OF NITRATE REDUCTASE FROM NINE-DAY-OLD SOYBEAN LEAVES.

Fraction	Volume	Total activity	Recovery	Protein	Specific activity
	ml.	*units*	*%*	*mg./ml.*	*units/mg. protein*
I. Crude extract	190	2774	....	8.10	1.8
II. First calcium phosphate gel eluate	38	1281	46	0.80	42.2
III. 0–40% ammonium sulphate precipitate	15	645	23	1.10	39.1
IV. Second calcium phosphate gel adsorption eluate	3	199	7	0.55	120.6

again was centrifuged for five minutes and the supernatant fluid discarded. The enzyme was eluted by mixing 38 ml. of 0.1 M pyrophosphate buffer at pH 7.0 with the gel and allowing it to stand for 10 minutes. The suspension was centrifuged for 15 minutes and the supernatant fluid (fraction II) collected. This fraction contained 46% of the total activity and was purified 24-fold (table I).

Ammonium sulphate (9.2 gm.) was added to 38 ml. of the first eluate (fraction II), the suspension was allowed to stand for 10 minutes, then was centrifuged for 10 minutes. The precipitate was dissolved in 15 ml. of cold 0.1 M phosphate buffer at pH 7.0. This solution is referred to as fraction III. Seven and one half milliliters of calcium phosphate gel were added to 15 ml. of fraction III. After 10 minutes, the mixture was centrifuged and the supernatant liquid discarded. The gel was washed with 7.5 ml. of cold 0.1 M phosphate buffer at pH 7.5, centrifuged for five minutes and the gel collected. Three milliliters of 0.1 M pyrophosphate buffer at pH 7.0 were mixed with the gel, allowed to stand for 10 minutes, centrifuged for 15 minutes, and the eluate (fraction IV) collected.

The data in table I show that this procedure resulted in a 67-fold purification of the enzyme, with recovery of 7% of the total activity. The minimum purification obtained in repetitions of this procedure was 44-fold with 11% recovery. Since relatively large quantities of enzyme were necessary, the first eluate (fraction II) was used in many of the experiments. The range in purification of this fraction was 18.5- to 27-fold, and its protein content ranged between 0.8 and 1.0 mg. per ml. Fraction IV contained 0.5 to 0.9 mg. protein per ml. The turnover number for this fraction is 2.4 moles of nitrate reduced to nitrite per mole of protein per minute, assuming a molecular weight for the enzyme of 100,000. The quantity of purified enzyme (fraction II or IV) used in experiments with the standard assay procedure contained 0.05 to 0.2 mg. of protein.

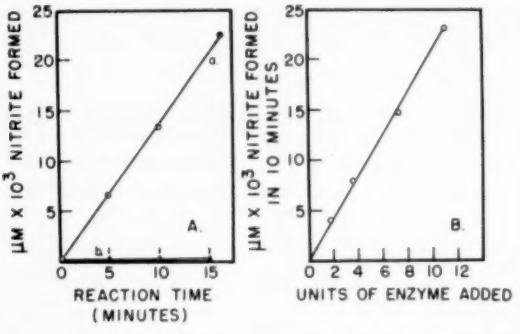

FIG. 1. A. Proportionality of enzyme activity with time. Procedure was that of the standard assay (see text) with exception of the time as indicated. The enzyme used was 6.7 units of fraction II (curve a) or this quantity boiled (curve b). B. Proportionality of activity with enzyme concentration. Procedure was that of the standard assay. Units of enzyme (fraction II) are indicated.

Tests of fractions II and IV for other enzymes indicated no diphosphopyridine nucleotidase activity (**16**), but very low glutathione reductase (**20**), and fairly strong cytochrome c reductase activities (**15**) were observed. The latter did not parallel nitrate reductase activity in the purification process, and it is concluded, therefore, that cytochrome c reductase and nitrate reductase are not the same enzymes. The inclusion of succinate in the reaction mixture with or without TPNH, under aerobic or anaerobic conditions, had no effect on the nitrate reductase activity. The purified enzyme (fraction IV) was very heat sensitive, as indicated by an experiment which showed 28, 46, 71, and 100% inactivation from five-minute exposures at 20, 30, 40, and 50° C, respectively. The activity was reduced 50 to 70% during storage periods of one week at – 15° C. As shown in figure 1, the activity was proportional to the quantity of enzyme added and also to the length of time reaction mixtures were incubated.

Requirements for optimum activity

pH optimum.—As indicated in figure 2, the pH optimum is at 6.0 with relatively high activity in the range 5.5 to 7.0. There was no marked difference in activity from use of phosphate as compared to acetate buffers on the acid side of the optimum or from phosphate and pyrophosphate buffers on the alkaline side of the optimum. When the soybean nitrate reductase was purified by ammonium sulphate fractionation only, the addition of pyrophosphate resulted in no stimulation of activity. In contrast, the Neurospora nitrate reductase (**22**), prepared by the same method, showed a striking response. Experiments reported in this paper were carried out at pH 7.0 for convenience.

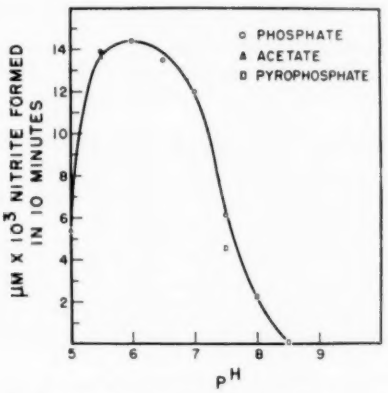

Fig. 2. Effect of pH on nitrate reductase activity. Procedure was that of the standard assay (see text) except that 42 μM of the buffers indicated were added. Six units of fraction IV were used as the enzyme.

Substrate concentration.—The final concentration of KNO_3 required to saturate the enzyme in presence of an optimum concentration of TPNH was 2×10^{-2} M (fig. 3). The concentration for one half maximal activity, which is a measure of the dissociation of the enzyme-nitrate complex (K_M) is 7.5×10^{-3} M, as determined from the saturation curve. The nitrate contamination usually found in TPNH was avoided by several reprecipitations of TPN in the course of its purification.

The curves in figure 4 show that TPNH or DPNH were almost equally effective as hydrogen donors for nitrate reduction. The maximum activity, in presence of an optimal concentration of nitrate and other known cofactors was obtained at a final concentration of 8×10^{-5} M for both reduced coenzymes. The concentration of TPNH required for one half maximal activity was 2.3×10^{-5} M and that for DPNH was 3.2×10^{-5} which would indicate a slightly greater affinity of the enzyme for TPNH than for DPNH. The

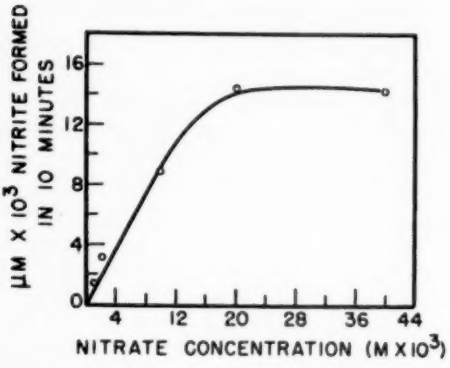

FIG. 3. Nitrate required for saturation of enzyme. Procedure was that of standard assay (see text) with exception of the final concentrations of KNO_3 used which are indicated. Seven units of fraction II were used as the enzyme.

differences, however, are of a magnitude where experimental error is probably an important consideration. The TPNH, prepared by chemical reduction with hydrosulphite (**32**), appeared to be as effective as enzymatically prepared TPNH, although it was less stable when stored at – 15° C.

INORGANIC IONS.—No consistent increase in activity could be obtained by preliminary incubation at 10^{-4} M final concentrations of $ZnSO_4$, $MnSO_4$, Na_2MoO_4, $Na_2B_4O_7$, $MgSO_4$, $FeSO_4$, $FeCl_3$, or $CuSO_4$. Attempts to dialyze the enzyme prior to incubation with these compounds resulted in complete

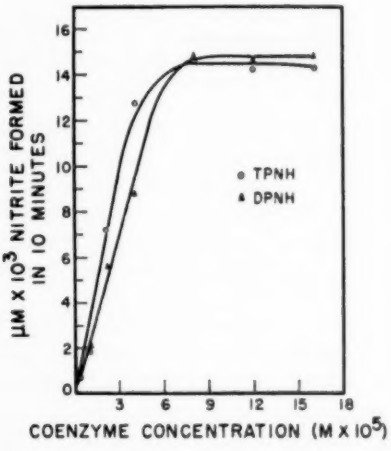

FIG. 4. Comparison of TPNH and DPNH as hydrogen donors in soybean nitrate reductase system. Procedure was that of the standard assay (see text) with the exception that TPNH and DPNH concentrations, on a final volume basis, were varied as indicated. The enzyme used was 7.3 units of fraction IV.

TABLE II

DISSOCIATION OF FLAVIN NUCLEOTIDE FROM ENZYME BY AMMONIUM SULPHATE PRECIPITATION.

Samples of enzyme fraction II	Units of enzyme		Nitrite formed in 10 minutes			
			Before precipitation		After two precipitations	
	Before precipitation	After precipitations	With FAD	Without FAD	With FAD	Without FAD
			$\mu M \times 10^3$			
A	10.0	9.5	20.2	19.6	19.1	14.1
B	16.0	13.0	32.2	30.3	25.9	15.8
C	7.8	6.0	15.6	14.1	12.1	5.8

inactivation which could not be reversed. There was no difference in nitrate reduction by this enzyme system under anaerobic, as compared to aerobic conditions.

Dissociation of flavin nucleotide prosthetic group

The enzyme activity of the first eluate (fraction II) was not appreciably affected by additions of FAD or FMN. A very marked response to FAD, however, was demonstrated after twice precipitating the enzyme with $(NH_4)_2SO_4$ (table II). Dissociation of the prosthetic group by this method was accomplished as follows: solid $(NH_4)_2SO_4$ was added to the enzyme solution (45% saturation), then after 10 minutes at 4° C, the suspension was centrifuged and the precipitate dissolved in the original volume of cold 0.1 M phosphate buffer at pH 7.0. The process was then repeated. Three different extracts obtained by this method from three preparations of fraction II

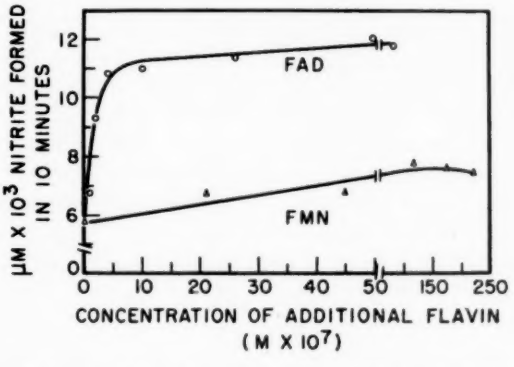

Fig. 5. Flavin nucleotide requirement of nitrate reductase. Procedure was that of the standard assay (see text) with the exception of the final concentrations of FAD or FMN indicated. All constituents except TPNH were incubated five minutes at 4° C prior to the nitrate reductase assay. Six units of preparation C (see text) were used as the enzyme.

contained 0.3 to 0.6 mg. protein per ml. They will be referred to as preparations A, B, and C.

The activity of the twice-precipitated enzyme (preparation C) as a function of FAD and FMN concentrations is illustrated in figure 5. A final concentration of 1×10^{-6} M additional FAD was sufficient for almost maximum activity. One half maximal reactivation of the enzyme was obtained with FAD at approximately 1×10^{-7} M final concentration and with FMN at about 3.7×10^{-6} M. The affinity of the enzyme, therefore, is much greater for FAD than FMN. These results show that the activity of the enzyme which was decreased approximately 50% by two $(NH_4)_2SO_4$ precipitations was completely restored by FAD and that FMN additions caused a very slight activation. The greater effectiveness of FAD in reactivating the nitrate reductase does identify the natural prosthetic group, however, the following experiments show that FAD is the nucleotide associated with the enzyme.

The flavin nucleotide associated with the twice-precipitated nitrate reductase (preparations A and B) was further identified as FAD by the method

TABLE III

FLUORIMETRIC ANALYSIS OF SOYBEAN NITRATE REDUCTASE FLAVIN NUCLEOTIDE.

Analyzed	Galvanometer deflections due to flavins		
	Flavin from boiled enzyme		FAD
	Preparation A	Preparation B	
Before hydrolysis (f_1)	2	3	11
After hydrolysis (f_2)	13	13	36
Per cent. flavin as FAD	98	89	81

of BURCH *et al.* (**4**), which is based on the increase in fluorescence obtained by hydrolyzing FAD to FMN and adenylic acid. Flavin nucleotides were liberated from the enzyme by heating 3.5 ml. aliquots at 100° C for five minutes in the dark. The supernatant liquids (collected by centrifugation) and a known sample containing 3.8×10^{-3} μM of FAD were hydrolyzed by incubating them in the dark with 100 μM phosphate buffer at pH 7.5 and 1.5 mg. of snake venom (5 mg. protein per ml.) containing pyrophosphatase, in a final volume of 5 ml. for 30 minutes at 37° C. The snake venom showed no fluorescence due to flavins. Control solutions (before hydrolysis, in table III) of the boiled enzyme and FAD were incubated with all constituents used with those hydrolyzed, except pyrophosphatase.

A marked increase in fluorescence due to flavin nucleotides was obtained by hydrolyzing all samples (table III). Since the fluorescence of FAD is 0.14 times that of FMN, the per cent. of FAD in a mixture is given by the following equation (**4**):

$$\text{Per cent. FAD} = \frac{f_2 - f_1}{0.86\ f_2} \times 100 \qquad (1)$$

where f_1 and f_2 are measures of flavin nucleotide fluorescence before and after hydrolysis, respectively. From the increase in fluorescence obtained by hydrolysis with nucleotide pyrophosphatase, it is calculated that 98, 89, and 81% of the flavins in preparation A, B, and the FAD solution, respectively, is accounted for as FAD. By use of the fluorescence data and that of a known riboflavin standard, it was calculated that preparation A contained 2.8×10^{-4} μM FAD per ml. and preparation B contained 2.6×10^{-4} μM per ml. (table IV).

Additional proof that the soybean nitrate reductase contained FAD was provided by its activity with the protein of D-amino acid oxidase specific for FAD (**33**). In this experiment, 2.6 ml. of the boiled preparation A and B adjusted at pH 8.5, and 0.2 ml. of the apoenzyme of D-amino acid oxidase were placed in the main compartment of a Warburg vessel. Two tenths milliliter of 0.33 M DL-alanine was placed in the side arm, and KOH in the center well. The mixture was preincubated for 30 minutes at 4° C, and oxygen uptake was determined at 30° C. The oxygen uptake of other reaction mixtures containing from zero to 2.5×10^{-3} μM of FAD, instead of the

TABLE IV

FLAVIN-ADENINE DINUCLEOTIDE CONTENT OF SOYBEAN NITRATE REDUCTASE.

Preparation	Method of analysis	
	Fluorimetric	d-Amino acid oxidase
	μM per ml.	*μM per ml.*
A	2.8×10^{-4}	2.3×10^{-4}
B	2.6×10^{-4}	1.6×10^{-4}

boiled enzyme preparations, was determined at the same time, in order to estimate the FAD content of the unknown samples. Preparations A and B contained 2.3×10^{-4} and 1.6×10^{-4} μM FAD per ml., respectively, as determined by this method. This compares favorably with the values for the corresponding samples determined by the previously described fluorimetric procedure (table IV).

Stoichiometry of reaction

In order to determine the stoichiometry of the nitrate reductase reaction, an experiment designed to measure concomitant disappearance of TPNH and formation of nitrite was carried out in spectrophotometric cuvettes with the approximate proportion of constituents used in the standard assay. Methylene blue was added to the solvent cuvette to compensate for the absorption due to the enzyme. At various intervals during the reaction period, aliquots of the reaction mixtures were removed from cuvettes and assayed for nitrite. As shown by the spectrophotometric measurements (fig. 6), there was a very rapid oxidation of TPNH in presence of the complete nitrate reductase system, none when the enzyme was omitted, and a fairly rapid endogenous rate when all constituents, except KNO_3 were present.

Since nitrite was formed in the complete system only, the endogenous rate (without nitrate) was caused by some factor other than nitrate. The observed values of nitrite formation and TPNH oxidation (difference between curve A and curve B of fig. 6) are reported in table V. These results are in agreement with the conclusion that nitrate reduction by this system proceeds according to the following equation:

$$NO_3^- + TPNH + H^+ \rightarrow NO_2^- + TPN + H_2O \qquad (2)$$

The appreciable endogenous disappearance of TPNH in the absence of nitrate suggests that the enzyme (fraction II) possibly contained a TPNH oxidase system, as described by Conn *et al.* (8). It is interesting that the optical density of the complete reaction mixture (curve A, fig. 6) increased

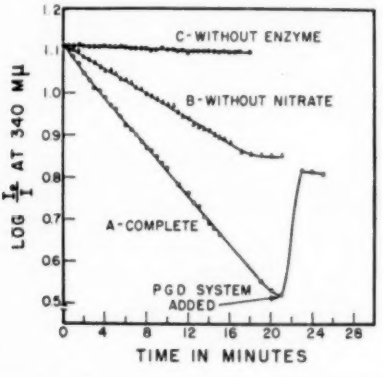

Fig. 6. Disappearance of TPNH in presence of soybean nitrate reductase system. Reaction mixtures contained the following, in a final volume of 3.5 ml.: curve A, 45 units of enzyme (fraction II), 625 μM phosphate buffer at pH 7.0, 0.01 μM FAD, 100 μM KNO_3, and 0.63 μM TPNH; curve B, the constituents added were the same as indicated for curve A except that KNO_3 was omitted; curve C, quantities of constituents were the same as indicated for curve A with the exception that the enzyme was omitted. After 21 minutes 0.02 ml. of purified phosphogluconic dehydrogenase and 47. μM of sodium phosphogluconate were added to the reaction (curve A). The phosphogluconic dehydrogenase contained 0.32 units of enzyme and 0.5 mg. protein.

to a level equal to that of the endogenous curve (without nitrate) when the phosphogluconic acid dehydrogenase system was added (after 21 minutes), indicating that some of the TPN had been destroyed. Otherwise, the absorption should have increased to the original starting value.

Effect of inhibitors

The sensitivity of the enzyme to various inhibitors suggests the presence of a metal constituent in the nitrate reductase system (table VI). The procedure in table VI was that of standard assay with the exception that inhibitors as indicated and all reactants except TPNH were incubated five minutes at 4° C prior to starting the reaction with TPNH. The enzyme

TABLE V

CONCOMITANT DISAPPEARANCE OF THE REDUCED DERIVATIVE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE CALCULATED FROM DIFFERENCES BETWEEN CURVES A AND B OF FIGURE 6 AND PRODUCTION OF NITRITE DETERMINED FROM ALIQUOTS OF REACTION MIXTURE (CURVE A, FIG. 6)

Time	TPNH disappeared	Nitrite formed
min.	$\mu M \times 10^3$ *per ml.*	$\mu M \times 10^3$ *per ml.*
0.0	0.0	0.0
2.0	5.4	6.0
4.0	10.1	13.5
6.0	18.0	15.5
8.5	22.3	25.5
10.0	23.5	28.4
13.0	30.6	32.0
15.0	37.7	40.0
21.0	53.3	56.0

consisted of five units of fraction II. KCN at final concentrations of 10^{-3} and 10^{-4} M inhibited the activity to an extent of 83 and 27%, respectively, and NaN_3 at these concentrations inhibited 98 and 39% respectively. A concentration of 2×10^{-4} M potassium ethyl xanthate inhibited the activity 49% and 10^{-4} M $CuSO_4$ inhibited 32%. There was no inhibition by 100% CO or 10^{-3} M NaF.

Results of an experiment showing reversal of *p*-chloromercuribenzoate inhibition of the enzyme activity by cysteine hydrochloride are recorded in

TABLE VI

EFFECT OF INHIBITORS ON SOYBEAN NITRATE REDUCTASE ACTIVITY.*

Inhibitor	Final concentration	Inhibition
	M	%
o-Phenanthroline	2×10^{-3}	10
aa-Dipyridyl	2×10^{-3}	10
Diethyldithiocarbamate	2×10^{-3}	23
Potassium ethyl xanthate	2×10^{-3}	56
Potassium ethyl xanthate	2×10^{-4}	49
Potassium ethyl xanthate	2×10^{-5}	29
Thiourea	2×10^{-3}	56
Thiourea	2×10^{-4}	29
Sodium fluoride	10^{-3}	0
Potassium cyanide	10^{-3}	83
Potassium cyanide	10^{-4}	27
Sodium azide	10^{-3}	98
Sodium azide	10^{-4}	39
Cupric sulphate	10^{-4}	32
Carbon monoxide 100% (in dark)	....	0
Hydroxylamine hydrochloride	10^{-3}	12
8-Hydroxyquinoline	2×10^{-3}	11

*No preliminary incubation.

table VII. The procedure used was as follows: reaction mixtures (final volume of 0.7 ml.) containing 50 μM phosphate buffer at pH 7.0, 10 μM KNO_3, four units of enzyme (fraction II) and concentrations of *p*-chloromercuribenzoate indicated in table VII were incubated five minutes at 4° C. Cysteine hydrochloride as indicated and an equivalent amount of Na_2CO_3 for neutralization were added to reaction 5 through 8 and water to maintain equal volumes in reactions 1 through 4. Reactions again were incubated five minutes at 4° C. Afterward 0.001 μM of FAD (as boiled pig heart extract), and 0.08 μM TPNH were added to all mixtures and the nitrate reductase assay was carried out in the usual manner.

Preincubating the enzyme with concentrations of 10^{-3} M and 10^{-6} M *p*-chloromercuribenzoate resulted in 94 and 51% inhibition, respectively. If the reaction mixtures containing *p*-chloromercuribenzoate were incubated a second time with cysteine hydrochloride (8.3×10^{-4} M) before assaying for nitrate reductase, the inhibition was reduced to 56% with concentrations

TABLE VII

INHIBITION OF NITRATE REDUCTASE BY *p*-CHLOROMERCURIBENZOATE AND REVERSAL BY CYSTEINE HYDROCHLORIDE.

Reaction	*p*-Chloromercuribenzoate concentration	Cysteine hydrochloride concentration	Inhibition
	M	*M*	%
1	10^{-3}	....	94
2	10^{-4}	....	90
3	10^{-5}	....	81
4	10^{-6}	....	51
5	10^{-3}	8.3×10^{-4}	56
6	10^{-4}	8.3×10^{-4}	15
7	10^{-5}	8.3×10^{-4}	2
8	10^{-6}	8.3×10^{-4}	0

of 10^{-3} M *p*-chloromercuribenzoate and no inhibition was observed where 10^{-6} M *p*-chloromercuribenzoate was used. These results strongly suggest the presence of a sulfhydryl group on the enzyme surface (**13**).

Photochemical reduction of nitrate

Many investigators have reported a pronounced effect of light on nitrate assimilation by plants. During the course of this investigation, it was observed that homogenates of soybean leaves harvested early in the morning showed very little capacity to reduce nitrate without the addition of TPNH or DPNH. However, homogenates of leaves that had been exposed to bright sunlight for periods of a few hours in most cases were very active in reducing nitrate in the absence of reduced coenzymes. This might be ascribed to the photochemical reduction of pyridine nucleotides by grana (**2, 31**), which was coupled with the nitrate reductase system.

The design and results of an experiment showing the photochemical reduction of nitrate by use of grana and nitrate reductase obtained from

soybean leaves are given in table VIII. Complete reaction mixtures 1 and 6 (final volume of 1.5 ml.) contained: 150 μM phosphate buffer at pH 7.0, 6 μM KNO_3, 0.2 μM TPN or TPNH as indicated, grana containing 0.11 mg. chlorophyll, and 15.6 units of fraction II (no FAD requirement) as the enzyme. Constituents omitted in other reaction mixtures are indicated. Quantities of KCl in the grana preparation or pyrophosphate in the enzyme extract were added to reaction mixtures not including grana or enzyme. Light reactions were carried out under an intensity of 1400 foot-candles and dark reactions were shielded with aluminum foil. All reactions were incubated in a water bath at 28° C.

As indicated, 6 μM of KNO_3 were added to each reaction mixture of 1.5 ml. final volume. This was only one fifth of the concentration used in the

TABLE VIII

PHOTOCHEMICAL REDUCTION OF NITRATE WITH NITRATE REDUCTASE AND GRANA FROM SOYBEAN LEAVES.

Reaction time	1 Nitrate reductase KNO_3 TPN Grana	2 KNO_3 TPN Grana	3 Nitrate reductase TPN Grana	4 Nitrate reductase KNO_3 Grana	5 Nitrate reductase KNO_3 TPN	6 Nitrate reductase KNO_3 TPNH Grana
min.			$\mu M \times 10^3$ *nitrite**			
			Light			
0	0.0	0.0	0.0	0.0	0.0	0.0
10	12.3	1.5	6.6	1.8	0.9	19.5
20	25.7	4.8	18.0	2.4	0.0	38.1
30	34.5	7.2	22.5	2.7	0.0	65.2
			Dark			
0	0.0	0.0	0.0	0.0	0.0	0.0
10	3.6	0.3	1.8	1.5	0.3	16.2
20	4.5	5.4	3.6	3.6	0.0	27.6
30	5.1	7.2	2.7	6.6	0.0	28.2

*Quantity of nitrite in entire reaction volume of 1.5 ml., determined from aliquots.

standard nitrate reductase assay. The smaller quantity was necessary because the concentration of nitrate required for optimum nitrate reductase activity resulted in a striking decrease in photochemical reduction of TPN. Washing soybean grana preparations resulted in a serious decrease of their photochemical activity, and therefore, unwashed preparations were used that contained small quantities of nitrate from the leaves.

A very definite photochemical reduction of nitrate was demonstrated (table VIII, reaction 1) in the light. The low rate of nitrate reduction observed in the dark indicates that the reaction mixture contained a weak system for reducing TPN that was not directly dependent upon light. The minute quantity of nitrite formed in reaction 2, containing no purified nitrate reductase, implies that the grana preparation contained a low con-

centration of nitrate reductase. The omission of nitrate from the system (reaction 3) resulted in a fairly high nitrite formation in the light, but very little in the dark. The grana preparation undoubtedly provided a supply of nitrate in this reaction. A small quantity of nitrite was formed in reaction mixtures without TPN (reaction 4) in both light and dark, indicating that the grana contained coenzymes or other substances effective in nitrate reduction. There was almost no endogenous nitrite formation in reaction 5 (without grana). It is interesting to observe that reaction 6, containing TPNH proceeded more rapidly in the light than the dark. It seems logical that in presence of active grana a greater proportion of TPNH would remain reduced in the light than in the dark, thus preventing the depletion of the TPNH supply by oxidation in the nitrate reductase system and other systems undoubtedly present in the grana. Light had no effect on the nitrate reductase system without grana. This experiment was repeated, using 0.45 μM of $NaNO_2$ in reaction mixtures, instead of KNO_3. Determination of nitrite in all reaction mixtures, after incubating for 30 minutes, showed no nitrite disappearance. These results, however, are not quantitative, since nitrite was formed in certain reactions from the nitrate associated with the unwashed grana. Since the enzyme activity (reactions 1 and 6 in the light) was proportional to the incubation time, it seems improbable that sufficient nitrite disappeared to affect the results appreciably. Repetitions of the experiment (table VIII) with different enzyme and grana preparations indicated the same trends of results as those reported.

Effect of nutrition on nitrate reductase activity

Soybean seedlings were grown for five days in triplicate cultures containing nutrient solutions with nitrogen sources as follows: (a) $Ca(NO_3)_2$, (b) $(NH_4)_2SO_4$, (c) without nitrogen. The enzyme activity of homogenates from the primary leaves of these plants was determined by the standard assay procedure, using hydroxylamine hydrochloride to inhibit partially the nitrite disappearance. Homogenates of all leaves, regardless of source of nitrogen provided, showed high nitrate reductase activity ranging between 8×10^{-3} and 1.1×10^{-2} μM nitrite formed in 10 minutes per mg. of dry leaf weight. Diphenylamine tests indicated the absence of nitrates in nutrient solutions without N or with $(NH_4)_2SO_4$, but a positive nitrate test was obtained with leaves of plants grown in these solutions. Therefore, the question of whether or not the enzyme is adaptive remains unanswered.

The nitrate reductase activities of homogenates of young soybean leaves from 30-day-old plants showing symptoms of Fe, Mn, Zn, B, Mo, and N deficiencies were compared with those of control plants. The variation of results from day to day was so great that no conclusions could be drawn. No such extreme variation in the enzyme activity of seedlings was observed.

Distribution of the enzyme

Results from a survey of the distribution of the nitrate reductase in a limited number of higher plant species is reported in table IX. The stand-

ard assay was used with the following exceptions: incubation times were as indicated; 0.5 μM $NH_2OH \cdot HCl$ was added to each reaction (0.5 ml. final volume) to partially inhibit nitrite disappearance; 0.2 ml. of homogenates was used as the enzyme. Equal concentrations of DPNH and TPNH were used. It was necessary to incubate reaction mixtures containing homogen-

TABLE IX

THE OCCURRENCE OF NITRATE REDUCTASE IN CERTAIN PLANT SPECIES.

Plant species		Nitrite formed in 1 hr.*		
		No coenzyme	TPNH	DPNH
	days	*$\mu M \times 10^3$ mg. dry wt.*		
Potato, variety Irish Cobbler, *Solanum tuberosum* L.				
Roots	30	0.0	1.7	1.2
Leaves	30	1.8	2.4	2.5
Barley, variety Alpha, *Hordeum vulgare* L.				
Roots	30	0.3	3.7	2.2
Roots	17	0.5	1.0	....
Leaves	17	1.2	1.3	....
Muskmelon, variety Honey Rock, *Cumcumis melo* L.				
Roots	30	0.0	2.2	1.3
Roots	17	1.8	4.0	....
Leaves	17	0.0	0.2	....
Wheat, variety Nittany, *Triticum vulgare* Vill.				
Roots	30	0.7	6.8	4.6
Tomato, variety Rutgers, *Lycopersicum esculentum* Mill.				
Roots	38	0.1	4.5	11.4
Roots	31	5.1	16.0	23.5
Corn, variety Lancaster Surecrop, *Zea Mays* L.				
Roots	28	0.2	1.2	1.5
Soybean, variety Odgen, *Glycine max.* Merr.				
Roots	17	0.0	1.5	....
Leaves (primary)	17	0.0**	0.6**	....
Leaves (young trifoliate)	17	6.2**	12.5**	....
Leaves (meristematic)	17	5.1**	12.0**	....
Leaves (young trifoliate)	30	1.0	1.3	....
Leaves (primary)	8	33.3**	63.7**	....

*Single determinations except soybean samples which are means of duplicate determinations.

**Incubation time was 10 minutes, results calculated on an hour basis.

ates of most plants for an hour, in order to obtain a quantity of nitrite that could be measured accurately. The results show that the addition of reduced coenzymes consistently increased quantities of nitrite accumulated. This is considered as strong evidence that the nitrate reductase purified from soybean leaves or a similar enzyme is present in at least six other plant species.

The enzyme appeared to be concentrated in the meristematic and young trifoliate leaves of 17-day-old soybean plants. The primary leaves of 17-day-old plants contained little enzyme, in contrast to a very high concentration in primary leaves of eight-day-old seedlings which suggests that the enzyme is concentrated in young, metabolically active tissues.

Nitrite disappearance

In studying the enzymatic reduction of nitrate to nitrite, it was necessary to determine whether or not nitrite disappeared in reaction mixtures. This could be determined with enzyme preparations free of nitrate, by substituting nitrite (1.5×10^{-2} μM) for nitrate in the standard assay procedure and determining the quantity that disappeared. By use of this method, no nitrite disappearance could be detected with the various enzyme fractions obtained in the purification. There was a small non-enzymatic disappearance of nitrite, however, if TPNH and nitrite were allowed to remain in contact with the acid sulfanilamide reagent for a few minutes before adding the solution of *N*-(1-naphthyl)-ethylenediamine hydrochloride reagent. This possibly was caused by deamination of the amino group of the adenine in TPNH.

In preliminary experiments it was found that a protein precipitate collected by 35% $(NH_4)_2SO_4$ saturation of the supernatant liquid from the first calcium phosphate gel adsorption would catalyze a slow disappearance of nitrite. In one experiment 6.8×10^{-3} μM nitrite disappeared in 20 minutes in the presence of the enzyme and TPNH, whereas none disappeared when TPNH was omitted in the reaction or when boiled enzyme was used. The nitrite reductase activity could be inhibited approximately 80% by adding a final concentration of 10^{-3} M $NH_2OH \cdot HCl$ to the reactive mixture. This concentration resulted in 12% inhibition of the nitrate reductase activity. Tests indicated no nitrite formation from incubations of $NH_2OH \cdot HCl$, TPN, and enzyme.

The nitrite reductase system indicated in soybean leaves also has been observed in washed homogenates of tomato roots. This system requires further investigation.

Discussion

The nitrate reductase purified from soybean leaves is similar to that found in Neurospora (**11, 22**). They are not identical, however, as indicated by a variety of evidence. The two enzymes have different pH optima, show marked differences in specificity for reduced coenzymes I and II, behave differently in the purification procedure, show different dissociation properties of the flavin nucleotide prosthetic group, and have different turnover numbers.

It was shown that the activity of the twice-precipitated soybean nitrate reductase was markedly stimulated by FAD, but responded very slightly to FMN. Tests with D-amino acid oxidase and fluorimetric analyses indicated

that enzyme solutions contained FAD and that 89 to 98% of the total flavin present in the enzyme extract was FAD. From this information and the data in table IV, it was calculated that the enzyme extract contained a maximum of 3.4×10^{-5} μM FMN per ml. This or greater quantities of FMN were not sufficient to produce an appreciable stimulation of the activity of the twice-precipitated enzyme in the standard assay procedure. In view of these facts, it seems conclusive that the natural prosthetic group of the enzyme is FAD and not FMN.

The strong inhibition of the activity by *p*-chloromercuribenzoate and reversal by cysteine hydrochloride suggest that a sulfhydryl group is involved in the active surface. This seems a probable explanation for the sensitivity of the enzyme to cupric ions. The pronounced inhibition by potassium cyanide, sodium azide, thiourea and potassium ethyl xanthate indicates the necessity of a metal for enzymatic activity. Since it is well known that deficiencies of Mo and Mn result in nitrate accumulation (**3, 5**), it has been suggested that one of these metals is involved. The answer to this problem, however, remains for future investigation.

The possible physiological significance of this enzyme is obvious. Reduced pyridine nucleotides necessary for nitrate reduction could be readily supplied by the large number of enzyme systems that reduce these coenzymes by oxidation of various substrates. Some of these enzymes that are known to be present in higher plants (**3, 30**) are hexose phosphate dehydrogenase, malic dehydrogenase, glutamic dehydrogenase, isocitric dehydrogenase, formic dehydrogenase, triosephosphate dehydrogenase, and alcohol dehydrogenase. The energy required for the production of TPNH or DPNH by these systems would be derived from the intermediates of carbohydrate metabolism, and, therefore, would offer a logical explanation for the disappearance of carbohydrates that has been observed (**9, 12**) to accompany nitrate reduction *in vivo*.

The energy for nitrate reduction, via reduced coenzymes I and II, is not necessarily derived from the oxidation of carbohydrate intermediates as was demonstrated by the photochemical reduction of nitrate by the following reactions:

$$H_2O + TPN \xrightarrow{\text{light and grana}} 1/2O_2 + TPNH + H^+ \qquad (3)$$

$$TPNH + H^+ + NO_3^- \xrightarrow{\text{nitrate reductase}} NO_2^- + TPN + H_2O \qquad (4)$$

Vishniac and Ochoa (**31**) used anaerobic conditions when reaction 3 was utilized in a coupled system to fix carbon dioxide. In the experiments reported in this paper, reactions 3 and 4 catalyzed by grana and nitrate reductase from soybean leaves, proceeded at a relatively rapid rate under aerobic conditions.

Results of these experiments offer a plausible explanation of the direct effect of light on nitrate reduction which has been reported by Bŭrstrom

(**6**). It is interesting to consider that a concentration of 0.02 M nitrate, that was optimum for nitrate reductase activity, resulted in approximately 75% inhibition of the photochemical reduction of TPN. This inhibitory effect may explain the observation of LUNDEGÅRDH (**18**) that leaves of nitrogen-deficient oat plants were much more active photosynthetically than leaves of comparable plants provided with adequate nitrate.

Summary

The purification and properties of an enzyme from soybean leaves is described which catalyzes the reduction of nitrate to nitrite by the oxidation of TPNH or DPNH. Evidence is presented that this or a similar enzyme is present in the six other higher plant species examined.

Experiments show that the enzyme is a flavoprotein with FAD as the prosthetic group. It is sensitive to heavy metal inhibitors, including potassium cyanide, sodium azide, thiourea and potassium ethyl xanthate. Inhibition by *p*-chloromercuribenzoate was reversed by cysteine hydrochloride, which suggests that a sulfhydryl group is present on the active enzyme surface. No inhibition was obtained by use of CO or NaF.

The pH optimum activity is at 6.0. No stimulation of activity is obtained by additions of $ZnSO_4$, $MnSO_4$, $Na_2B_4O_7$, Na_2MoO_4, $MgSO_4$, $FeSO_4$, $FeCl_3$, or $CuSO_4$ at final concentrations of 10^{-4} M.

Determinations of the TPNH that disappeared in the reaction and the quantities of nitrite that appeared concomitantly, indicate that one mole of nitrate is reduced by one mole of the reduced coenzyme.

By use of grana and purified enzyme from soybean leaves, the photochemical reduction of nitrate has been demonstrated. The significance of these results are discussed in relation to previous reports of the effect of light on nitrate assimilation.

No measurable quantity of nitrite disappeared in reaction containing purified nitrate reductase fractions. Disappearance, however, was observed in certain homogenates and other preparations. Nitrite disappearance could be partially inhibited by the addition of 10^{-3} M final concentration of $NH_2OH \cdot HCl$.

Preliminary evidence indicates that TPNH at least is involved in nitrite as well as nitrate reduction.

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CARBOHYDRATE METABOLISM IN THE TOMATO FRUIT AS AFFECTED BY POLLINATION, FERTILIZATION AND APPLICATION OF GROWTH REGULATORS[1]

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Introduction

While there is considerable information on the morphological effects from pollination and fertilization and application of growth-regulating substances on fruit development, our knowledge of the physiological aspects related thereto is still very meager. In a general way, it has been observed that, as a result of sexual reproduction (syngamy), the over-all metabolism of ovaries and associated tissues is greatly stimulated resulting in diversion of food to these organs (**25**). This stimulus from reproduction can be initiated and maintained in many plants by the timely application of an appropriate synthetic plant growth-regulator (**8**). The present paper deals with one physiological aspect, carbohydrate metabolism, as affected in fruit shortly after pollination, fertilization, or application of growth regulator.

Marked changes in carbohydrate content in ovaries after pollination were observed in orchids by HSIANG (**12**), and SESHAGIRAH (**32**) noted an increased starch synthesis in the ovaries, receptacles, and pedicels immediately after fertilization which also has been pointed out as a common occurrence by MARRÈ (**16, 19**). The excision of young seeds usually leads to a reduction in starch and sugar content in associated tissues (**17, 19**) with a corresponding increase in amylase activity. These facts indicate quite definitely that the seeds have a controlling influence on carbohydrate metabolism of the fruit and adjoining parts of the plant. An effect on carbohydrate content is usually obtained by the application of a growth regulator as is evidenced by the results secured with roots (**4**), leaves (**22, 23**), stems (**2, 3, 5, 6, 35**), young floral organs (**16**), and stomata (**20**). MARRÈ (**16**) was able to substitute the application of indoleacetic acid for pollination and fertilization as regards an effect on amylase activity and synthesis of starch reserves in the fruit of Erodium and Pelargonium. That auxins are produced in fruits has been shown by GUSTAFSON (**8**), WITTWER (**37**), HAAGEN-SMIT *et al.* (**9**), NITSCH (**30**) and others.

In the light of this evidence, the following inferences appear probable: (a) The fertilized ovules are powerful centers for the production of native auxins, which regulate the carbohydrate metabolism in the fruit. (b) Growth regulators effect changes in carbohydrate metabolism similar to those

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brought about by pollination and fertilization. It was thought desirable to study further the analogy between pollination-fertilization and treatment with growth regulator in their bearing on change in carbohydrate content, with special reference to the crucial early stages of fruit development.

Experimental procedure

The experiments were conducted with the tomato (*Lycopersicum esculentum* var. Marglobe) grown in soil in large containers and kept in a well-lighted, thermostatically controlled greenhouse. The plants were allowed to grow to a large size and were vigorous. Fruit setting was prevented by defloration, excepting where specified otherwise. The experimental treatments were as follows: (a) Flowers pollinated, at noon on the first day of anthesis. (b) Flowers emasculated the day before anthesis. Two thirds of each style was removed and pure lanolin applied at noon of the following day. (c) Treatment as in (b) but a growth regulator was incorporated into the lanolin.

The regulators used were *p*-chlorophenoxyacetic acid (CPA) and the ethyl ester of indoleacetic acid (EtIA). The first compound has been shown

TABLE I

EFFECTS OF POLLINATION AND GROWTH REGULATORS (*p*-CHLOROPHENOXYACETIC ACID, CPA; ETHYL ESTER OF INDOLEACETIC ACID, EtIA) ON GROWTH AND CARBOHYDRATE CONTENT OF YOUNG TOMATO FRUITS.

Age and treatment	Average weight	Reducing sugars	Sucrose	Starch	Soluble sugars	Starch and soluble sugars
	mg.	*gms./100 gm. fresh wt.*				
Controls, at anthesis	9.5	0.10	0.96	0.091	1.06	1.15
Controls, not pollinated, 3 days after anthesis	9.5	0.10	0.81	0.087	0.92	1.00
Controls, pollinated, 3 days after pollination	74	0.49	0.32	0.860	0.81	1.67
CPA, 10^{-3} M, 3 days after hormone treatment	108	0.91	0.50	0.910	1.41	2.32
EtIA, 10^{-3} M, 3 days after hormone treatment	97	0.63	0.53	1.100	1.16	2.26
EtIA, 10^{-4} M, 3 days after hormone treatment	79	0.54	0.34	0.82	0.89	1.71
EtIA, 3×10^{-5} M, 3 days after hormone treatment	13	0.160	0.68	0.107	0.84	0.90
EtIA, 10^{-5} M, 3 days after hormone treatment	10	0.12	0.79	0.088	0.91	1.00
Controls, pollinated, 8 days after pollination	3760	1.09	0.203	1.42	1.30	2.72
CPA, 10^{-3} M, 8 days after hormone treatment	4050	1.50	0.21	1.17	1.71	2.88
EtIA, 10^{-3} M, 8 days after hormone treatment	3800	1.19	0.26	1.46	1.45	2.91

TABLE II

EFFECT ON CARBOHYDRATE CONTENT AND ON GROWTH OF TOMATO FRUIT WHEN TREATED WITH DIFFERENT CONCENTRATIONS OF THE ETHYL ESTER OF INDOLEACETIC ACID EtIA INTERVAL AFTER TREATMENT WAS 48 HOURS.

Treatment	Average weight	Reducing sugars	Sucrose	Starch	Soluble sugars	Starch and soluble sugars
	mg.		*gm./100 gm. fresh wt.*			
Controls, at pollination	10.5	0.081	0.92	0.047	1.00	1.047
Controls, not pollinated, 2 days after anthesis	11	0.077	0.89	0.042	0.97	1.009
Controls, 2 days after pollination	16	0.090	0.73	0.505	0.82	1.32
			No other fruits present			
EtIA, 10^{-3} M	25	0.200	0.61	1.62	0.81	2.43
EtIA, 4×10^{-4} M	26	0.170	0.62	1.31	0.79	2.00
EtIA, 10^{-4} M	25	0.115	0.59	1.20	0.71	1.91
			Five to seven other fruits present			
EtIA, 10^{-3} M	22	0.150	0.67	1.01	0.82	1.83
EtIA, 4×10^{-4} M	21	0.133	0.68	0.89	0.81	1.70
EtIA, 10^{-4} M	19	0.095	0.70	0.57	0.79	1.37

by MURNEEK (**24**) to be potent in the stimulation of tomato fruit setting, and the second has been found to be an unusually active naturally occurring derivative of indoleacetic acid (**31**). In either case the substance was first dissolved in ethanol and then incorporated into lanolin in concentrations given in the tables (tables I and II). The alcohol was removed at 40° C. The lanolin paste was applied to the cut surface of the style by means of a micrometer controlled syringe fitted to a medium-sized needle which made it possible to control the quantity of paste used at 0.01 ± 0.001 ml.

Treatments were performed at noon to minimize as much as possible the fluctuations in carbohydrate content due to variations in light and temperature. The plant material for analyses was collected on the same day, or two, three, or eight days after treatment. Each sample consisted of 8 to 14 average fruits. The material was fixed and preserved in alcohol, extracted and cleared by the usual procedures, and the carbohydrates were determined by the NELSON colorimetric technique (**28**).

Results

The results of carbohydrate analyses are presented for unpollinated ovaries treated with lanolin, ovaries stimulated by pollination and fertilization, ovaries treated with growth regulators, and ovaries treated with growth regulator in the presence of other developing fruits. Growth of the ovaries was measured by average changes in fresh weight. The data presented in

tables I and II and figures 1 and 2 show that, if pollination is prevented, no significant change in starch and reducing sugar content occurs during a period of two to three days after anthesis. The small decrease in sucrose concentration probably indicates a loss due to respiration. Considering the relative stability in carbohydrate metabolism of unpollinated ovaries, it is possible to make a comparative study of the effects of pollination and fertilization and chemical treatment.

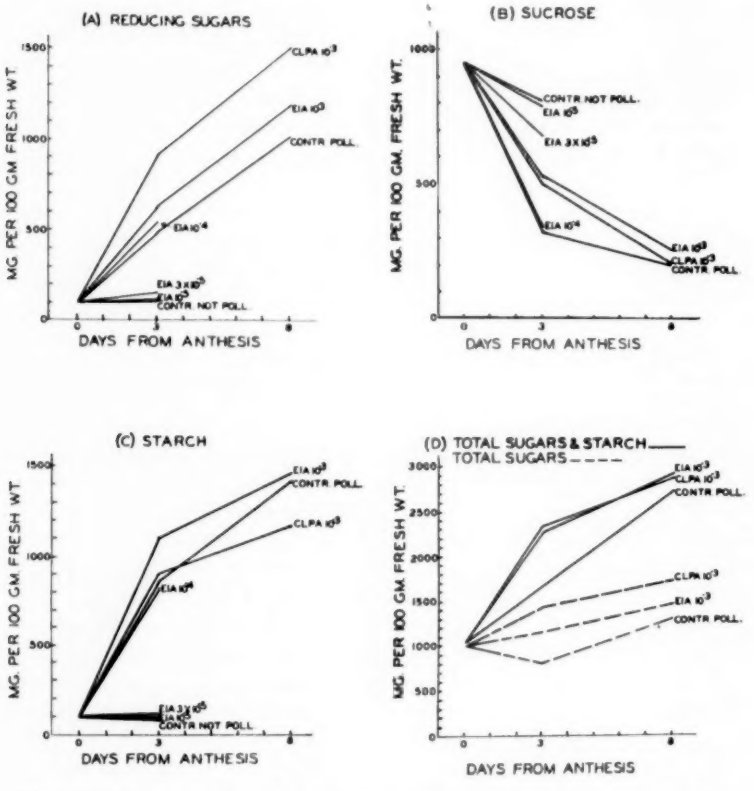

FIG. 1. Effects of pollination and growth regulators (*p*-chlorophenoxyacetic acid, CPA; or ethyl ester of indoleacetic acid, EtIA) on carbohydrate content of young tomato fruits. A, reducing sugars. B, sucrose. C, starch. D, total sugars and starch.

As early as 48 hours after pollination, there was a marked increase in starch content of the ovaries, a slight increase in reducing sugars and a noticeable decrease in sucrose, which, as was noted before, had occurred to some extent also without pollination. From the third to eighth day after pollination there was a further striking increase (500 to 1100%) in reducing sugars and starch. A further decrease of sucrose, not as marked as on the

first days but still noticeable, was also observed. As a consequence of this alteration in sucrose content in comparison to reducing sugars, the concentration of the total sugars decreased after pollination until the third day and then increased rapidly. Total carbohydrate (starch plus sugars) content increased immediately after pollination or treatment.

Both *p*-chlorophenoxyacetic acid (CPA) and the ethyl ester of indoleacetic acid (EtIA) induced carbohydrate changes in young tomato fruits

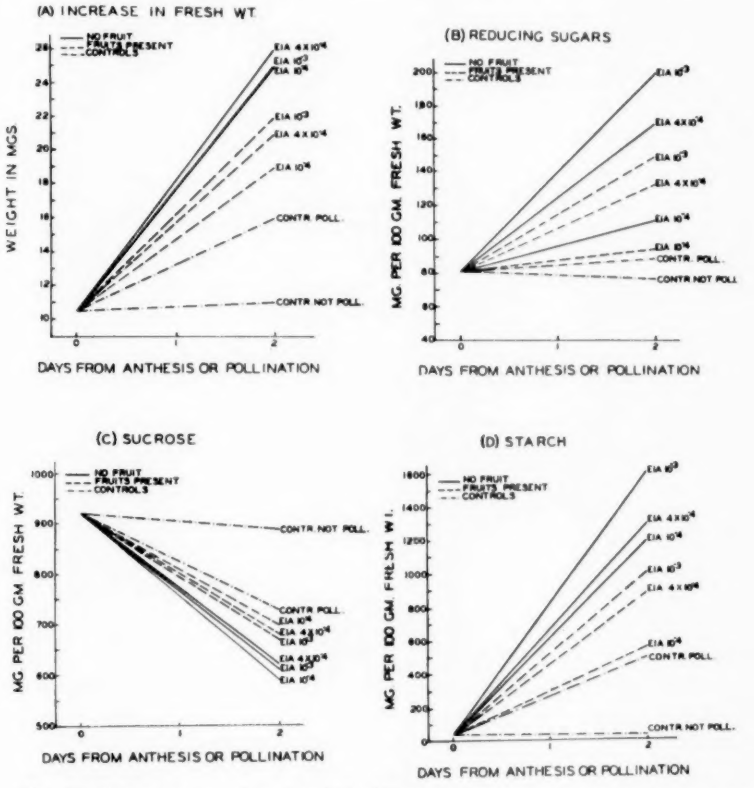

FIG. 2. Effects on growth and carbohydrate content of young tomato fruits, during a two-day interval, when treated with different concentrations of the ethyl ester of indoleacetic acid (EtIA). A, increase in fresh weight. B, reducing sugars. C, sucrose. D, starch.

that were very similar to those brought about as a result of pollination and subsequent fertilization. Reducing sugars and starch increased and sucrose decreased. The chief difference between the effects of pollination and fertilization and of an external supply of a growth regulator was a more rapid response to the latter, which however showed a tendency to disappear with time. CPA was possibly more active than EtIA in increasing the reducing

sugar content but was less active in stimulating starch synthesis. When EtIA was applied in concentrations greater than 10^{-4} M, the effect on carbohydrate metabolism appeared to be proportional to the concentration, but a concentration of 3×10^{-5} M was practically inactive. This observation suggests the probable existence of a threshold concentration as regards the effects of EtIA on carbohydrate metabolism, at least in this instance.

The fruit set and development of the tomato is affected by the presence of older fruits on the plant, whether the growing fruits are in the immediate vicinity of the young ones (fig. 3) or at some distance (**26**). In this test, older fruits were absent or five to seven fruits approximately three weeks old were present. These fruits were borne at other positions on the plant than the experimental ovaries. It is evident from table II and figure 2 that in the presence of fruits the increase in reducing sugars and starch and the

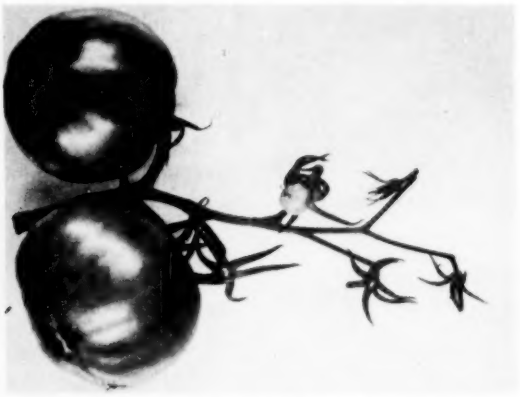

FIG. 3. Inhibition of young tomato fruits by two older fruits growing rapidly.

decrease in sucrose was considerably less, and there was likewise a somewhat similar reduction in increase in fresh weight of the fruit.

During the first 48 hours the growth of the fruit, as indicated by change in fresh weight, was increased as a result of pollination but much more from application of EtIA. It was also significantly greater in the absence than in the presence of other large fruits on the plant (table II). After fertilization (from the third day on) which usually has a more marked stimulation on growth than pollination alone, there was still a noticeable difference in size between the fruits induced naturally and those induced by the hormone in favor of the latter. By the eighth day this difference had decreased considerably. It would seem that stimulation from an external supply of a hormone was initially greater than from pollination and fertilization but that it decreased more with time. The native auxin produced as a result of fertilization seems to have a continuous and probably increasing effect on metabolism and growth (**23**).

Discussion

It is a well established fact that indigenous auxins are of importance for fruit setting and development (**8, 25**). In the present investigation it has been shown that a close similarity exists in general carbohydrate metabolism between pollinated and fertilized ovaries and ovaries treated with a hormone. The fact that the carbohydrate changes are less marked after pollination than with auxin treatment but that after fertilization they soon reach the same level both in the fertilized and in the treated ovaries seems to indicate that during pollen germination and pollen tube growth, auxin is released in smaller amounts than after fertilization, which in the tomato occurs about two days after pollination (**13**). It has been demonstrated that in corn, following fertilization, a peak in native auxin concentration is reached in about 10 to 15 days (**37**). No such maximum has been established in the tomato.

The main features of carbohydrate metabolism as a result of pollination and fertilization or treatment of tomato ovaries, were the over-all increase of the total carbohydrate concentration and the passage to a different equilibrium between the different carbohydrates through the initiation of starch synthesis, the decrease of sucrose, and the striking increase of reducing sugars. These results are in good agreement with the analytical records for flowers, the naturally set young fruit of *Pyrus malus* (**27**), and the observations of carbohydrate changes after pollination or chemical treatment of orchids (**12**). Following pollination a rapid starch synthesis has been observed in ovaries of orchids (**32**) and ovaries of Aralia, Erodium and Pelargonium (**14, 16**).

It would seem to be desirable to emphasize some general aspects of the action of hormones, native or externally supplied, on the carbohydrate content of the ovaries. In consideration of the relationship between activation of carbohydrate metabolism and growth of the fruit, the following points may be recognized: A significant increase in ovary growth occurred only in presence of those concentrations of an applied hormone that gave a stimulation in starch and reducing sugar accumulation. Growth was closely proportional to changes in carbohydrate content. When carbohydrate response to hormone treatment was reduced, because of the presence of other rapidly developing fruits on the plant, the growth of the ovaries was also reduced. This parallelism suggests a probable interdependence between the growth of the fruit and its carbohydrate metabolism.

It would seem logical to assume that the observed carbohydrate changes were not induced by stimulation of growth but that probably it was the other way around. When a hormone concentration too high to give increased stimulation of growth was applied, it was still effective in bringing about the characteristic changes in carbohydrates. Growth of the ovaries, therefore, probably was affected by the changed carbohydrate metabolism in the tissues until the appearance of some limiting factor of a different nature. The

importance in metabolism of other substances such as various forms of nitrogen compounds and the soil nutrients must not be denied however.

The over-all increase of carbohydrate concentration in the growing ovary appears in complete agreement with the general occurrence of high sugar concentrations in or near points of high metabolic activity. A similar behavior was found by MARRÈ (**15**) to be common to floral buds, young fruits, immature seeds, and root apices, and it is interesting to observe that in all these organs the removal of the probable centers of auxin production was followed by a clear decrease in sugar concentration.

The mechanism of such an accumulation of carbohydrates is still an open problem. In the case of the rapidly growing ovary, one would expect a decrease of the initial concentration as a consequence of water uptake during growth and metabolic utilization of carbohydrates for respiration, which is noticeably increased after pollination or auxin treatment (**12, 34, 36, 39**), and for synthetic purposes. The fact that the opposite condition is found, *i.e.*, carbohydrate concentration increases, shows that in the developing ovary a powerful mechanism is working to accelerate the rate of sugar intake in the growing cells. This mechanism, as far as is known, could operate in two different ways. One seems to consist of the action of the young fruit in mobilizing reserves in other regions of the plant. The existence of this effect of mobilization at a distance and its correlative nature was pointed out by MURNEEK (**26**) and in its enzymatic aspects, by MARRÈ and by MARRÈ and BARLASSINA (**17, 19**). But a simple mobilization effect, if active in increasing the availability of carbohydrates, does not explain their preferential accumulation in the growing ovary. In fact, in these tomato plants the following concentrations in soluble carbohydrates were found in the following organs: leaves 0.71% (fresh weight basis); young stems, 0.78%; old stems, 0.92%; fruit pedicels, 0.56%; fruits seven days after pollination, 1.45%; fruits seven days after treatment with *p*-chlorophenoxyacetic acid, 1.75%. This preferential accumulation in the ovary tissues suggests that in these tissues after pollination or auxin treatment the power to obtain sugars from the vascular system is increased. A similar conclusion was reached by MASKELL and MASON (**21**) in order to explain the movement of sugars and nitrogen to the young fruits of cotton. These authors speak of an increase in permeability to these compounds in the growing cells. It would appear preferable to speak of an increase in absorption power rather than of an increase of permeability, as plant cells are, as a rule, only very slightly permeable to sugars, so a rapid uptake of the latter is, without doubt, to be considered an active physiological process.

A good, even if indirect, evidence that auxin increases the absorption power towards sugars in the ovary tissues may be found in the fact that excised tomato ovaries, cultured in a medium containing sucrose, thus precluding a mobilization effect, react to auxin with an increase in growth (**29**) and with the initiation of starch synthesis (present evidence), since growth and synthesis of starch appear closely related to an efficient carbohydrate

uptake. Another interesting aspect of the changes in carbohydrate metabolism in the pollinated or auxin treated ovaries consists of the initial sharp decrease in sucrose which could be due to a preferential utilization of this sugar to the hexoses for anabolic purposes as well as for respiration. A similar interpretation was proposed by CHRISTIANSEN and THIMANN (**5**) for the decrease in sucrose without a decrease in reducing sugars in sections of pea stems cultured in a medium containing auxin. Though other interpretations such as the partial conversion of the disaccharide to reducing sugars are possible, a preferential utilization of sucrose in the first stages of growth could be explained by the possibility that it undergoes phosphorolysis, yielding highly active hexose phosphate, and by its higher energy content (**7, 11, 33**).

As far as the two most important features of carbohydrate response, the increase in total carbohydrate concentration and the synthesis of starch, are concerned it is interesting to consider them in connection with the increase in respiration, which apparently is a normal consequence of pollination and fertilization or auxin treatment. The accumulation of carbohydrates, as pointed out before, probably involves an increase in the power of absorption of sugars in the tissues of the ovary. An increased power of absorption requires an increase in expenditure of energy, which must be supplied by respiratory metabolism. The rate of respiration appears to be correlated also with the initiation of starch synthesis in the activated ovaries. As phosphorylase is present in tomato fruit (unpublished results) and seems to be most important for starch synthesis in higher plants (**10, 18, 38**), it appears reasonable to assume its vital role in this case. But phosphorolytic synthesis of starch implies, as the first step, the phosphorylation of hexoses through a reaction of the type:

$$\text{Hexose} + \text{ATP} \longrightarrow \text{Hexose phosphate} + \text{ADP}.$$

The production of adenosine triphosphate (ATP) is conditioned by the rate of respiration, and with this reasoning, the main responses of carbohydrate metabolism to pollination and fertilization or auxin treatment appear closely related to the increase in respiration which probably is the basis for the initiation of ovary activation and fruit development.

Summary

In the pollinated and fertilized ovaries of the tomato the first stages of development are accompanied by significant changes in carbohydrate metabolism. Two main aspects of these changes are recognizable: (a) Alternations in the equilibrium between the different carbohydrates occur, indicating modifications in the enzymatic pattern. Conspicuous features of this aspect are a rapid increase in starch, a simultaneous decrease in sucrose and a progressive rise in reducing sugar concentration. (b) A marked increase of the capacity to absorb, retain, and concentrate carbohydrates occurs. The total concentration of carbohydrates (soluble and insoluble) rose steadily after

pollination but especially after fertilization. The concentration of soluble sugars, after a slight initial decrease, soon reached an amount far above that present at anthesis.

Application of the growth regulator, *p*-chlorophenoxyacetic acid or the ethyl ester of indoleacetic acid, to the cut surface of the style of emasculated flowers produced effects strikingly similar to those induced by pollination and fertilization including starch synthesis, a decrease of sucrose, and an increase of reducing sugars. Carbohydrate accumulation during the first two days was noticeably higher in treated ovaries which may be related to the comparatively slow release of auxin after pollination with a gradual and more prolonged increase after fertilization.

The degree of modification in carbohydrate metabolism in the unpollinated ovaries was in proportion to the concentration of the growth regulator applied. At the lowest threshold concentration there was a minimal and parallel response for both induction of parthenocarpy and effect on carbohydrate metabolism. At the highest concentration used the growth regulators, while ineffective for further stimulation of growth of ovaries, still stimulated accumulation of starch and reducing sugars.

In presence of older developing fruit on the tomato plant, a treatment with a growth regulator had a reduced effect on both the changes in carbohydrates and the rate of fruit development.

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FACTORS AFFECTING COTYLEDONAL CRACKING DURING THE GERMINATION OF BEANS (*PHASEOLUS VULGARIS*)

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While studying cotyledonal abnormality an unusual type of injury was observed in snap beans which had been carefully hand-picked and hand-shelled. The injury consisted of one or more transverse breaks which extended partially or completely across the cotyledon, severing the vascular elements, figure 1. Not only was the movement of materials into the seedling impeded by the breaks but the cotyledons remained swollen often preventing the shedding of the seed coat, figure 2. The fissures also provide a seat of infection for decay organisms.

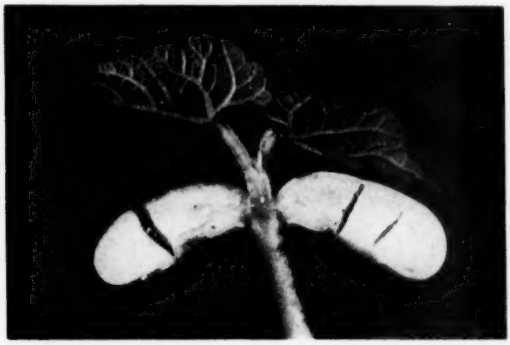

FIG. 1. Seedling showing cotyledonal fissures which developed during germination.

In a recent study of mechanical injury to seed beans (**1**) injury was classified into two categories: visible or external and invisible or internal, of which the latter was detected by a viability test. Transverse cotyledonal breaks were attributed to mechanical injuries during the processing of the seed. SHULL and SHULL (**5**) observed internal cracking of pea cotyledons due to uneven swelling. They explained an apparent increase in the rate of water absorption as due to cavities formed by the breaking but did not relate it to germination. MILLER (**3**) showed by x-ray photographs that lima beans absorb water through the vascular bundle of the hilum. It then goes to the chalaza and around the seed primarily through the vascular elements of the testa. The seed coat was found to be impermeable until wetted from beneath.

The present study investigates whether or not cotyledonal cracking takes place during germination and if so under what conditions. Seed characteristics as they affect cotyledonal cracking and related problems are also considered.

Materials and methods

The experimental beans were grown in the field at Urbana, Illinois, during the summer of 1950. At maturity, the seeds were harvested and shelled by hand, and then kept in the laboratory for about two months. Seven varieties and strains which had shown a variation in cracking of cotyledons were selected. They include Cherokee wax, 49-3 (Eastern States Farmer's Exchange), Commodore, Top Crop, Rival, B1229-1-2-6 (Regional Vegetable Breeding Laboratory) and B1762 (Regional Vegetable Breeding Laboratory) with respective percentages of cracking of 0, 1.3, 5.3, 13.5, 15.3, 38.7 and 48.3.

FIG. 2. Seedling with cotyledons broken during germination and seed coat adhering when ends of cotyledons failed to shrink.

Except where otherwise indicated, germination tests to determine cotyledonal cracking were made using 100 seeds of each treatment in a greenhouse maintained at 65 to 75° F. The seeds were planted in steam sterilized sand and covered about three fourths of an inch deep. The cotyledons were usually observed for cracking as soon as the old seed coats had shed. In a few cases, however, the seed coats were carefully removed for the examination.

Absorption of water was determined by placing seeds in distilled water at the rate of 100 seeds per 150 ml. of water and then removing them at intervals, depending upon the rates of absorption. The seeds were dried quickly between pads of absorbent cotton covered with cheesecloth and weighed. Unless otherwise stated, rates of absorption were measured at 20° C and calculated as percentage increase in weight during the period of observation.

Results

The testae were carefully removed from a large number of seeds of Top Crop which is subject to cotyledonal cracking and Cherokee which is resistant, and none were found with cracks when examined under a stereoscopic microscope. One hundred cotyledons of each variety were then placed on moist filter paper in covered Petri dishes at room temperature. Within an hour marked cracking occurred in the cotyledons of both varieties (fig. 3). Cherokee is resistant to cracking during germination but became susceptible when the seed coats were removed and water could be absorbed freely. This suggested that resistance to cracking depended upon the rate of water uptake and not upon a difference in the imbibitional properties of the cotyledons.

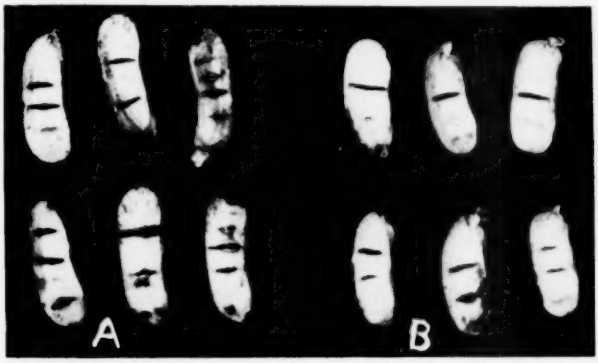

FIG. 3. Cotyledonal fissures resulting from rapid water absorption on moist filter paper after removal of the testae. The resistant variety Cherokee (A) cracked as readily as the susceptible strain 49-3 (B) under these conditions.

To check these points a number of experiments on the absorption of water were made. Seeds of B1229-1-2-6, Top Crop, 49-3 and Cherokee were soaked and the rates of uptake of water measured. After 30 hours the seed coats were carefully removed and the cotyledons observed for fissures. The data (fig. 4) show the rates of absorption by the strains to be in the order of B1229-1-2-6 > Top Crop > 49-3 > Cherokee. As indicated by wrinkling of seed coats the first two began absorbing water within a few minutes after immersion. About three hours were required for all of the seeds of 49-3 to begin and several hours before any of the seeds of Cherokee began, and then the water moved very slowly around the cotyledons of this variety as indicated by wrinkling of the seed coats. Strain B1229-1-2-6 and Cherokee with highest and lowest rates of absorption had respectively highest and lowest percentages of seeds with cracked cotyledons, 84 and zero. Although Top Crop absorbed water more rapidly than 49-3, it had only 59 as compared to 64% of seeds with cracked cotyledons. This is perhaps due to the delayed

absorption in 49-3 and to its longer cotyledons, which were subject to greater tensions as a result of differential swelling.

To determine if imbibitional properties of the cotyledons might in part account for the differences in rates of absorption by varieties, the testae were carefully removed from 50 seeds of each Cherokee and 49-3 and the excised cotyledons immersed in water. They were weighed at intervals of 30 minutes for five hours and then observed for cracks. The cotyledons of both Cherokee and 49-3 imbibed water rapidly and were severely cracked, the percentages being respectively 84 and 96. When the testae of the former were being removed, it was noted that they adhered tenaciously to the cotyledons, which may explain the slow wrinkling and movement of water into the seeds of this variety.

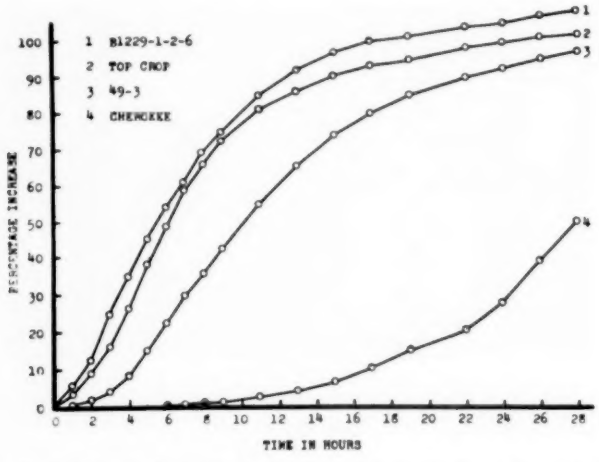

FIG. 4. Rate of water absorption by bean seed as percentage increase in weight.

Since the moisture content of the soil may be expected to affect the rate of absorption and consequently the cracking of cotyledons, three flats were filled with sand having respective moisture contents of 1.96, 3.52, and 13.59%. Each flat was planted with Top Crop and B1229-1-2-6 in alternate rows. The flats were then placed on a greenhouse bench and covered with glass over which paper was laid. After three days the covers were removed and all of the flats were watered uniformly. The data in table I show that lower soil moisture during the initial stage of germination resulted in decreased cotyledonal cracking. In strain B1229-1-2-6 it varied from 11 to 65%, while in Top Crop it ranged from 10 to 32. The data indicate that the severity of cracking may be increased when susceptible varieties are planted in wet soils.

To determine the effect of temperature on the relation between rate of absorption and cracking of cotyledons, Rival beans were soaked and the rates of absorption measured at 10, 20, and 30° C. At the end of seven

TABLE I

COTYLEDONAL CRACKING OF BEANS DURING GERMINATION IN SAND OF DIFFERENT MOISTURE CONTENTS.

Moisture content of sand	Cotyledonal cracking	
	B1229-1-2-6	Top crop
1.96	11	10
3.52	45	18
13.59	65	32

hours the testae were removed and the cotyledons examined for fissures. There was an increase in rate of absorption with temperature but a decrease in cotyledonal cracking. The percentages were 76, 70, and 60 for the respective temperatures. This experiment was repeated with germinating seeds. A one-gallon container filled from a lot of uniformly moist sand and covered to prevent loss of moisture was placed in each of three chambers maintained at 10, 20, and 30° C respectively. As soon as soil temperatures became constant, seeds of the Rival variety were planted in each container. When germination began, the seeds were taken from the sand, the seed coats removed and the cotyledons observed for fissures. The results show the same inverse relationship between cotyledonal cracking and temperature but more strikingly than in the previous experiment. The percentages of seeds with cracked cotyledons were 65, 54, and 28 for the respective 10, 20, and 30° C temperatures. The data indicate that cracking would be increased by low soil temperatures.

Because of the low relative humidities in the laboratory where the beans had been stored since harvest, the cotyledons were rather brittle. Under such conditions the seed might be predisposed to cotyledonal cracking. Therefore, the effect of seed conditioning on subsequent cracking of cotyledons during germination were investigated. Samples of 100 seeds of Cherokee, B1762 and Commodore with two 100-seed lots of Top Crop, five lots in all, were placed in each of six desiccators. Relative humidities of approximately 95, 75, 56, 35, 15, and 0% were maintained in the desiccators by

TABLE II

PER CENT. RELATIVE HUMIDITY OF SEED STORAGE, MOISTURE CONTENT OF BEANS AND COTYLEDONAL CRACKING DURING GERMINATION.

Storage humidity	Moisture content of Top Crop	Cotyledonal cracking			
		B1762	Cherokee	Commodore	Top Crop
95	28.7	37.9	0.0	1.0	0.0
75	16.2	25.8	0.0	0.0	0.0
56	11.5	15.5	0.0	0.0	0.0
35	8.8	37.7	0.0	2.1	3.1
15	5.7	87.8	0.0	2.0	22.4
0	3.3	73.3	0.0	3.2	19.1

L.S.D. at 5% Level, 12.29; at 1% Level, 16.99

means of saturated salt solutions of disodium phosphate, sodium chlorate, calcium nitrate, chromic oxide, lithium chloride and anhydrous calcium chloride. The desiccators were covered but not sealed and placed in a constant temperature chamber maintained at 20° C.

One of the two lots of Top Crop seed in each desiccator was used as a control and weighed initially and then weekly until equilibrium was reached. After 31 days the moisture content of these controls was then determined by drying in a ventilated oven at 105° C. The remaining lots in the six desiccators were planted in sand filled flats in a randomized test with four replications of each treatment. The flats were watered uniformly and as needed until the seed germinated. Then the cotyledons were observed for fissures.

The moisture content of the Top Crop beans as shown in table II ranged from 28.7 to 3.3%. Even after storage at the range of relative humidities,

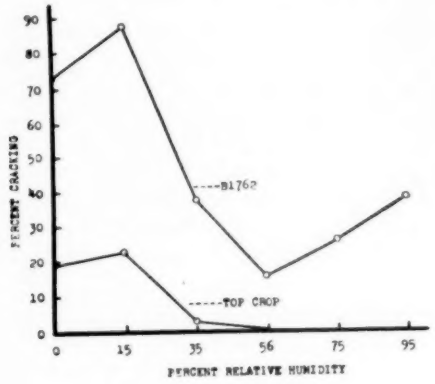

FIG. 5. Effect of storage humidity on cotyledonal cracking during the germination of beans.

all treatments of strain B1762 showed cotyledonal cracking ranging from 15.5 to 87.8% during germination. The percentage of cracking was lowest after storage at 56% and highest after storage at 15% relative humidity. Statistically significant cotyledonal cracking occurred in Top Crop beans after storage at low humidity, but not at the higher ranges and none occurred in Cherokee beans after storage at any of the six humidities. Although the percentage of cotyledons with fissures was low in Commodore beans after storage over anhydrous calcium chloride, the cotyledons tended to break off during germination.

Storage at low humidity caused some of the Cherokee beans to become hard (impermeable to water) and others to germinate slowly and unevenly. The data indicate that seed conditioning in beans with respect to moisture content may be important before planting or making a germination test, but the same conditioning may not be best for all varieties. When the controls

of Top Crop beans which had been stored at 95% relative humidity were dried rapidly in a ventilated oven at 105° C, the outer surfaces of the cotyledons cracked badly because of the more rapid shrinkage of the drying surfaces of the cotyledons. This indicates how internal cracking may result from unequal distribution of water in the cotyledons.

Germination was strikingly affected by the moisture content of the seed at the time of planting (fig. 5). Seed of B1762 from the 95% humidity chamber showed some decay, while the seed of Cherokee from this chamber germinated best. On the other hand, after storage over anhydrous calcium chloride germination of the B1762 variety was slow and uneven. Many of

FIG. 6. Germination of bean varieties after storage at different humidities. Varieties: A, B1762; B, Cherokee; C, Commodore; D, Top Crop. Relative humidity: 1, 95%; 3, 56%; 5, 75%; 6, 0%.

these seeds absorbed water but failed to germinate; others remained impervious to water. A similar observation on the effect of storage at low humidity on beans was made by NUTILE and NUTILE (4) and GLOYER (2).

Summary

Varieties of snap beans showed marked differences in susceptibility to cotyledonal cracking during germination. A high incidence of cracking was found in strains B1762, B1229-1-2-6, Rival, and Top Crop. No cracking was observed in the variety Cherokee except where the testae were broken. Susceptibility to cracking was associated with seed coat permeability and rapid imbibition of water. A rapid rate of water uptake apparently causes differential swelling in the cotyledons. Tensions arise that often result in transverse fissures.

Cotyledonal cracking was found to vary in relation to soil moisture during germination at 20° C, indicating that a high incidence of cracking may be expected when susceptible strains are planted in wet soils.

The relation between water absorption and cotyledonal cracking was altered by temperature. As temperature increased between 10 and 30° C, the rate of water uptake also increased but cracking decreased. More coty-

ledonal breakage may therefore be expected when susceptible varieties are planted in wet soils during a period of low temperatures.

The relative humidity at which seed beans are held for a period before planting may affect their susceptibility to cotyledonal cracking during germination. In the most susceptible strain tested, B1762, the maximum cotyledonal breakage occurred after storage at a relative humidity of 15%, a minimum breakage occurred at 56% and cracking was higher after storage at higher humidities.

From these experiments it seems likely that some of the bean seed injury attributed by other investigators to mechanical means may have occurred during germination.

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ACCUMULATION AND DISTRIBUTION OF MOLYBDENUM IN THE COTTON PLANT[1]

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Introduction

In recent years it has been established that molybdenum (Mo) plays an important role in the metabolism of higher plants. ARNON (**1**) working with barley noted that molybdenum, when added to cultures containing an ammonium salt as the source of nitrogen, stimulated growth. In a later investigation ARNON and STOUT (**2**) reported molybdenum as being essential for the normal growth and development of tomatoes. They stated that 0.01 p.p.m. of molybdenum was required to supply the needs of young tomato plants. The role molybdenum plays in the fixation of nitrogen by *Azotobacter chroococcum* had been reported earlier by BORTELS (**4**).

That molybdenum can be toxic to higher plants when supplied in quantities greater than needed for normal growth was established by WARINGTON (**8**). In experiments conducted on several species she reported characteristic toxicity symptoms due to excesses of molybdenum. ARNON and STOUT (**2**) report that 10 p.p.m. of molybdenum in the nutrient solutions caused injury to young tomatoes. BRENCHLEY (**5**) noted that plants were seriously injured with small doses of molybdate, in certain light and fen soils, while with heavier doses they were killed at an early stage. The inclusion of peat in the soil mixture usually resulted in a reduction of molybdenum toxicity. BARSHAD (**3**) stated that legumes tend to absorb more molybdenum than do nonlegumes when grown on soils containing available molybdenum in a concentration of a few parts per million.

The following investigation sheds some light on the absorption of molybdenum by cotton, its distribution within the plant, and the tolerance of cotton to relatively high concentrations of molybdenum in sand cultures.

Materials and methods

To facilitate digestion of the plant material, oven dry samples (1 to 2 grams) were placed in a muffle furnace and ashed at 250° C for two hours. The temperature was then raised to 500° C and the samples were allowed to remain an additional two hours. The ash was quantitatively transferred to Pyrex beakers (250 ml.) and 10 ml. of concentrated nitric acid and 2 ml. of 70% perchloric acid were added. The mixture was evaporated to dryness and 2 ml. of 30% hydrogen peroxide was added. Again the mixture was

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evaporated to dryness and 70 ml. of distilled water was added and the mixture boiled for two minutes. While hot, 10 ml. of concentrated hydrochloric acid was added and the solution filtered. On cooling the volume was brought to 100 ml. Aliquots of this solution were transferred to separatory funnels (130 ml.) and the colorimetric determination of molybdenum was conducted according to the method of EVANS *et al.* (**6**).

Cotton seeds, variety Stoneville 2B, were germinated in vermiculite and uniform seedlings were transplanted (January 20, 1951) into washed creek sand in two-gallon glazed pots. The seedlings were supplied one half of a liter of Hoagland's solution (**7**) daily. During this phase of the experiment each pot was thoroughly leached with deionized water at weekly intervals. Thirty-one days after transplanting, the various molybdenum treatments were started. The plants were thinned to one per pot, and five groups, containing five plants each, were randomized on the greenhouse bench. Molyb-

TABLE I

HEIGHT, FRESH WEIGHT AND DRY WEIGHT OF COTTON PLANTS 79 DAYS OLD AS INFLUENCED BY VARIOUS LEVELS OF MOLYBDENUM NUTRITION.

Mo in nutrient solution	Height	Fresh weight of whole plant	Dry weight of plant parts				
			Leaves	Stems	Roots	Bolls	Total
p.p.m.	*cm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
0	117.8	583.6	37.9	45.3	10.62	41.5	135.3
5	109.2	583.2	37.5	39.6	8.79	42.5	128.4
15	117.0	614.0	39.5	43.8	9.97	43.1	136.3
25	104.8*	525.6*	35.4	36.4*	8.56	35.5	115.9*
35	101.4**	478.6**	31.2**	31.5**	7.39**	34.6	104.8**
L.S.D. .05	11.1	71.6	4.8	8.4	2.10		17.7
L.S.D. .01	15.3	98.6	6.2	11.6	2.89		24.3

* Significant at 5% level.
** Significant at 1% level.

denum as $Na_2MoO_4 \cdot 2\,H_2O$ was added to Hoagland's solution in concentrations of 0, 5, 15, 25, and 35 p.p.m. Each plant received one liter of its respective nutrient solution per day. In order to prevent the accumulation of nutrients, the sand cultures were leached with deionized water and with nutrient solution three times weekly. In addition to sand cultures, one series of five plants was grown in Houston Black Clay. These plants received no molybdenum other than that contained in this soil.

Results

At the time of harvest (79 days after transplanting) both the 25 and 35 p.p.m. molybdenum levels significantly reduced height and fresh and dry weight measurements (table I). The differences between the 0, 5, and 15 p.p.m. treatments were within experimental error. At this date no differences in yield due to molybdenum treatment were noted. Symptoms of molybdenum toxicity other than growth reduction in the aerial portions of

the plant were few. In the 25 and 35 p.p.m. treatments a few of the lower leaves developed a golden color and by the time of sample collection some of these had shed. An examination of the roots showed a progressive change in color with increasing molybdenum content in the substrate. The color of the roots varied from a pale yellow for the zero level of molybdenum to orange for the 35 p.p.m. treatment.

Several weeks after starting the molybdenum treatments small necrotic spots appeared on the lower leaves of several plants. It was suspected that the necrotic spots were caused by some pathogen, and subsequent investigation proved *Ascochyta gossypii* Syd. to be the causal organism. Surveys to determine the extent of the disease within the experiment were made 65 to

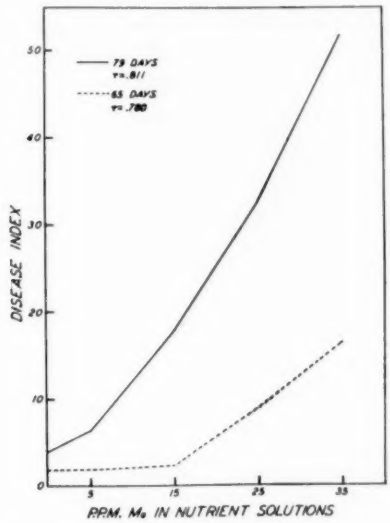

FIG. 1. The incidence of ascochyta leaf spot (*Ascochyta gossypii* Syd.) on cotton as influenced by molybdenum supply.

79 days after transplanting. Leaves infected with the causal organism were divided into four groups depending upon the severity of the infection. The infection of leaves with five or fewer spots were classified as light, 5 to 10 spots per leaf as medium, 10 to 20 as heavy, and above 20 as advanced. Numerical values of 1, 2, 3, and 4 in that order were given to the above classifications. On each plant the number of leaves within each of these classifications was determined and this sum was multiplied by its respective classification number. A summation of these values gave the disease index value for each plant. Figure 1 shows the relationship between the occurrence of Ascochyta leaf spot and the molybdenum level of the substrate. At both the 65- and 79-day surveys the correlation between disease index and molybdenum treatment was highly significant. It is also noted

from figure 1 that the intensity of the disease increased with age of plants as well as with molybdenum supply.

The results of chemical analysis for molybdenum make it apparent that cotton readily absorbs molybdenum when supplied as sodium molybdate. Table II presents the molybdenum content of leaves, stems, roots, and fruiting bodies as influenced by the molybdenum content of the substrate. The leaf samples consisted only of leaf blades; stems included the main stem, all branches and petioles; the fraction designated roots was composed of the tap root and larger secondary roots as pulled from the sand; squares, flowers and bolls were included in the fruiting bodies fraction. In all treatments the leaf fraction was highest in molybdenum content, ranging from 3 p.p.m. for the zero level to 1585 p.p.m. for the 35 p.p.m. treatment. The stems and roots were of approximately equal content, containing about one fifth as much Mo as did the leaves, while the fruiting bodies contained slightly less.

TABLE II

ACCUMULATION AND DISTRIBUTION OF MOLYBDENUM (MO) IN THE COTTON PLANT AS AFFECTED BY SUPPLY. RESULT AS P.P.M. DRY WEIGHT BASIS.

Mo supply	Leaves	Stems	Roots	Fruiting bodies
p.p.m.				
Houston Clay	3.4	2.4	0.7	0.8
0	3.0	1.0	0.4	0.4
5	316.2	68.4	72.2	59.8
15	898.0	188.6	171.2	144.8
25	1336.4	242.2	265.8	217.8
35	1585.2	304.4	319.8	253.8
L.S.D. .01	136.8	35.4	34.8	28.3
r* .01	.981	.971	.940	.985

*r values calculated from 25 pairs.

The plants grown in the Houston clay compared very favorably in molybdenum content with the zero sand culture series.

The data of table II indicate that molybdenum accumulation by the cotton plant is a curvilinear function of the Mo content of the substrate. Highly significant positive correlation coefficients were obtained for each plant fraction when molybdenum content was compared with substrate level.

Discussion

The results of this study show that cotton is able to absorb and accumulate molybdenum in relatively large amounts from nutrient solutions which contain only a few parts per million of this element in an available form, such as $Na_2MoO_4 \cdot 2\,H_2O$. In this experiment, plants were harvested 79 days after transplanting and at that time had received the respective molybdenum treatment for 48 days. The 35 p.p.m. molybdenum level caused a reduction of 18.07% in fresh weight. When determined on a dry weight basis this treatment was responsible for a reduction of 22.54% in growth. In view of the high accumulation of Mo, which reached a value of 1585

p.p.m. in the leaves, this reduction seems small and indicates a rather high tolerance of cotton towards this element. No differences were noted in boll production between the various treatments; however, it must be remembered that these plants were harvested at 79 days, and yield data taken at this stage does not necessarily give an accurate indication of the yield at maturity.

BARSHAD (3) reported the total molybdenum content of 22 plant species when grown on soils containing from 3 to 6 p.p.m. Mo in the soluble form. The plants analyzed varied in molybdenum content from 5 p.p.m. for *Sorghum halepense*, to 220 p.p.m. for *Vicia americana*. Although the comparison is not a direct one, the total Mo content of the above ground portion of the cotton plant was 143 p.p.m. when grown in sand cultures containing 5 p.p.m. Mo. This level compares very favorably with that of legumes, which, according to Barshad are accumulators of molybdenum. When Barshad added molybdenum, as ammonium molybdate, at rates of 10 and 50 p.p.m. to a soil normally containing .03 p.p.m. soluble Mo, the leaves of *Lotus corniculatus* were found to contain as high as 517 p.p.m. of this element. In comparison cotton leaves were found to contain 1585 p.p.m. Mo when the plants were grown in sand containing 35 p.p.m. Mo. It is recognized that some fixation of molybdenum may have occurred in the soil and that the soluble content of Mo was somewhat below the level applied; however, the evidence as presented here makes it clear that cotton too can be classified as an accumulator of molybdenum.

The infection of cotton by *Ascochyta gossypii* Syd. under the conditions of this experiment was noted to be directly correlated with increasing levels of molybdenum in the substrate. The high Mo content of the cotton leaves may have been responsible for a decrease in the physiological resistance of cotton to this disease or the trend noted in this experiment may be indicative of a high molybdenum requirement of *Ascochyta gossypii*. Since the disease incidence and molybdenum treatments went hand in hand it is impossible to determine which of these was responsible for the reduction in vegetative growth. BARSHAD (3) noted that the molybdenum content of *Lotus corniculatus* did not influence yield; however, as previously noted, ARNON and STOUT (2) reported injury to young tomato plants with as little as 10 p.p.m. Mo in the nutrient solution. In the investigation reported in this paper the molybdenum treatments were started 31 days after the seedlings were transplanted. The tolerance of well established cotton plants towards Mo is not necessarily an indication that seedlings will respond in the same manner. From investigations now in progress it is evident that cotton seedlings are more sensitive towards Mo and exhibit typical symptoms of toxicity when grown in Houston Black Clay containing 25 p.p.m. Mo.

Summary

Cotton absorbs and accumulates molybdenum readily from nutrient solutions which contain sodium molybdate. The amounts absorbed by cotton compare favorably with those of legumes which are considered to be accumu-

lators of this element. The leaves of cotton contained approximately five times as much molybdenum as the stems and roots. The fruiting bodies contained slightly less molybdenum than the stems and roots.

The incidence of Ascochyta leaf spot was found to be correlated with molybdenum level of the substrate. Reductions in both the fresh and dry weights of the cotton plant were also noted at the higher levels of molybdenum; however, it is not known whether the reduced vegetative growth was the result of molybdenum toxicity or was due to the increased infection of Ascochyta leaf spot at the higher molybdenum levels.

The writer is indebted to Mr. Luther S. Bird of the Department of Plant Physiology and Pathology, Texas Agricultural Experiment Station, for his identification of the organism *Ascochyta gossypii* Syd.

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THE USE OF C^{14} IN LOCATING GROWTH REGIONS IN THE CELL WALLS OF ELONGATING COTTON FIBERS

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It has been established that differential, or regional, elongation of plant cells occurs in many instances (**1, 2, 3, 7, 8**). This differential elongation in some cases involves the elongation of only the tip of the cell. Examples of cells which exhibit tip growth are root hairs (**2, 3**), flax fibers (**8**), and pollen tubes (**7**). In the present study the possibility of tip growth occurring in the elongating cotton fiber was studied by a procedure involving the incorporation of C^{14} in the cellulose portion of the elongating cell wall. The C^{14} was introduced when elongation of the fibers was in progress, and distribution of the radioactive carbon in the cell wall was determined by preparing radioautographs of the fibers.

Materials and methods

Cotton of the variety Delta Pineland was grown for the experiments in a greenhouse. The C^{14} was supplied in the form of barium carbonate obtained from the Isotopes Division of the Atomic Energy Commission at Oak Ridge.

The CO_2 generator and photosynthesis chamber constructed were similar to those described by NOGGLE and BOLOMEY (**4**), except that no electrometer was included. The photosynthesis chamber, large enough to hold a flowering cotton plant, was constructed of two bell jars placed bottom to bottom. In generating the CO_2, the $BaC^{14}O_3$ was diluted with enough ordinary $BaCO_3$ to produce an atmosphere of 5% CO_2 in the photosynthesis chamber. A slurry was made by adding water to barium carbonate in the generator. After placing the plant in the chamber and sealing it, the generator and chamber were evacuated to approximately 12 cm. of mercury. At this point the carbon dioxide was generated by adding an excess of H_2SO_4. After generation of the CO_2, sufficient vacuum existed within the chamber to draw air through the generator into the chamber. After flushing out the generator, the chamber was sealed and the plant inside was illuminated for 24 hours with six 100-watt fluorescent bulbs approximately three feet from the plant. At the end of this period, the atmosphere of the chamber was swept into CO_2-free alkali. Then the plant was removed and placed in the greenhouse for further growth.

The cellulose of the fibers was purified by the method of WISE *et al.* (**9**) for the purification of cellulose in wood. The fibers were refluxed in ethanolamine for 24 hours instead of six hours as recommended (**9**) since it was

found earlier that a longer period of refluxing was necessary to remove the abundant protoplasm of the elongating fibers (**5, 6**). Some fibers were treated only by boiling them for a few minutes in 5% oxalic acid, a treatment which dissolves the pectic outer coat of the cotton fiber and facilitates separating and straightening the fibers.

Radioautographs of the fibers were made on Eastman Kodak Autoradiographic Plates, type No Screen, which are recommended for radioautographs of beta particles. These were obtained in the size of one by three inches, the same size as ordinary microscope slides. The fibers to be radioautographed were placed on a microscope slide, dried, and placed against the autoradiographic plates, fiber against emulsion. The plates were exposed to the radioactive fibers for 30 days, removed and developed in a M-Q developer at the recommended temperature for five minutes.

Results

Carbon dioxide containing C^{14} was introduced into two cotton plants by the above described procedure. One fourth millicurie of C^{14} was given to the first plant. At the time this plant was supplied with the C^{14}, August 21, 1951, there were two bolls on the plant. The flowering date of the older boll, in which fibers were differentiating, was July 18; the flowering date of the younger boll, in which fibers were still elongating, was August 10. After the return of this plant to the greenhouse for further growth, a third boll appeared September 10. The fibers of all these bolls were allowed to mature before they were collected. Preliminary determinations of radioactivity were made by placing the crude fibers under a Geiger-Muller counter tube and showed that fibers from all three of the mature bolls were radioactive. After purification of the fibers to remove substances other than cellulose, the cellulose portions of the fibers were found still to be radioactive. The second plant treated was given one half millicurie $C^{14}O_2$ on January 11, 1952. At this time there was on the plant a single boll whose flowering date was December 31. This boll was about one half of the mature size; the fibers were approximately one half of the full length and in the process of elongating. After the radioactive carbon was administered, the boll was allowed to grow until January 19, at which time it was collected. At this time the boll was enlarging, indicating that the fibers were elongating and that no secondary wall formation had yet occurred. Preliminary determinations of radioactivity with a Geiger-Muller tube showed the crude fibers to be radioactive.

Radioautographs were made of untreated fibers containing protoplasm and of cellulose-purified fibers collected from the first plant. Enough radioactivity was present in a single mature untreated fiber collected from this plant to produce an image on the photographic plates used. Fibers from the same boll were purified by the ethanolamine treatment; a radioautograph was made of one of these cellulose-purified fibers. These radioautographs revealed that the basal ends of both the unpurified and the cellulose-purified

fibers had more radioactivity than the free ends. These radioautographs, because of the faint images produced, are not suitable for reproduction. Radioautographs were attempted using single fibers collected from the second plant while they were elongating. It was not possible, due to the low activity of the fibers, to produce a visible image on the plate in 30 days when a single cellulose-purified fiber was used. As an alternative to this procedure, a group of elongating fibers, attached at their bases to part of the seed coat, were carefully arranged parallel to one another on a slide and used for making radioautographs. The sample used to make the radioautograph, figure 2, was given only a preliminary treatment of boiling in oxalic acid which was necessary to separate and straighten the fibers. The radioautograph, figure 3, is of a mass of elongating fibers purified so that only the cellulose portion remains.

Discussion

The conclusions drawn from this study depend on two assumptions: one, that cellulose is deposited in the primary wall of the cell only during cell elongation and the other, that there is little or no exchange of carbon atoms between the cellulose already formed and other carbon compounds in the plant. These assumptions are based in part on general observations indicating that cellulose is a very stable product of metabolism in the higher plants. Apparently higher plants seldom, if ever, break down true cellulose once it is deposited in the cell wall. It is possible that carbon atoms in cellulose might be exchanged for new carbon atoms. However, it does not seem likely that the rate of exchange would be great enough to affect the results of the experiments conducted. In addition to these general observations there is specific evidence (**5**, **6**) that the percentage of cellulose, on a dry weight basis, is constant during the elongation of cotton fibers, and that

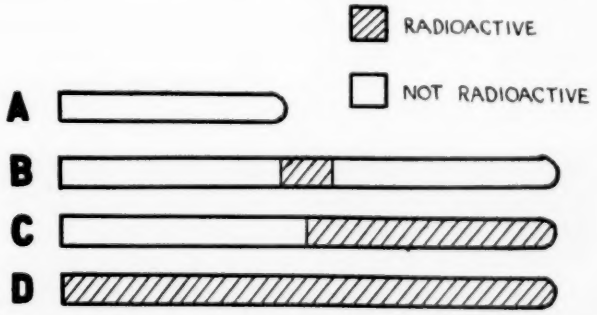

FIG. 1. Possible distribution of radioactive carbon in the cellulose portion of elongating cotton fibers. A. Length of fiber when C^{14} is administered. B. Distribution of radioactivity in elongating fiber if tip growth occurs and transfer of C^{14} to fiber is of short duration. C. Distribution of radioactivity in elongating fiber if tip growth occurs and transfer of C^{14} is of long duration. D. Distribution of radioactivity in elongating fiber if entire cell wall elongates.

FIG. 2. Radioautograph of elongating fibers of cotton containing C^{14}. The fibers were treated with oxalic acid in order to arrange them parallel to one another on the photographic plate. The knob at one end is of a portion of the seed coat to which the fibers are attached. Slightly enlarged.

the cellulose portion of the primary wall is structurally similar regardless of the age of the fibers.

It is to be expected that the more mobile compounds of the cell, as well as the cellulose, would be made radioactive, and that the entire elongating cell would contain C^{14}. However, if the assumptions made above are correct, only the portions of the cell wall which were constructed after the introduction of C^{14} would possess a radioactive cellulose framework. Hence, if the cell were as long as shown in figure 1 A when the C^{14} was introduced and only the tip of the cell elongated, the radioactive cellulose in the primary wall of a fully elongated cell should be that shown in figure 1 B or 1 C. If the C^{14} were transferred to the elongating fiber for only a short period, a radioactive band of cellulose should be formed as shown in figure 1 B. However, if the C^{14} continues to be deposited in growing fibers long after its original introduction into the plant, the pattern shown in 1 C is what would be expected. Since one boll on the first plant that was supplied C^{14} possessed radioactive fibers which began their growth 20 days after C^{14} was introduced into the plant, the C^{14} must have been transported into the fibers more than 20 days after its original introduction into the plant. Therefore, the radioactive pattern in 1 B would not be expected, while the pattern in 1 C would be possible if tip growth occurs. On the other hand, if the entire

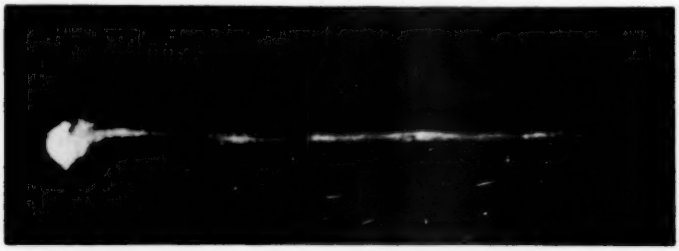

FIG. 3. Radioautograph of cellulose-purified elongating fibers arranged parallel to one another. The knob at one end is of a portion of the seed coat to which the fibers are attached. Slightly enlarged.

fiber elongates after the C^{14} is introduced, all of the cellulose framework of the primary wall should be radioactive, as in 1 D.

The results obtained by making radioautographs of the elongating fibers before extraction of the non-cellulose portions (fig. 2) show the entire length of the fiber to be radioactive, as would be expected since all of the protoplasm should be radioactive. However, that the cellulose frameworks of the elongating cells are also seen to be radioactive throughout their length (fig. 3) must be due to the fact that cellulose synthesis has taken place in all parts of the fibers after introduction of the C^{14}. From the latter observation it is concluded that the elongating fiber of cotton does not grow exclusively at the tip, but instead elongates along the entire length of the fiber.

That there was considerably more radioactivity in the fibers that were allowed to mature before collection indicates that the transfer of C^{14} into the growing fiber occurs for some time after its introduction into the plant as $C^{14}O_2$. The radioactivity of mature fibers whose formation was not initiated until after the C^{14} was introduced also shows that deposition of C^{14} in cell walls can occur long after the radioactive carbon is taken up by the plant. Radioautographs of both unpurified and cellulose-purified mature fibers show that these fibers are also radioactive throughout their length, as would be expected. More radioactivity is present in the basal portions of these fibers than in the apical portions.

Electron microscope studies of elongating fibers (**5, 6**) have shown that the structure of the tip of elongating fibers is very similar to the structure of the intermediate and basal portions of the fiber, as far as the cell wall is concerned. The presence of cellulose microfibers in the tip of the fiber as well as in other portions may be taken as an indication that the tip functions in the same way in elongation as does the rest of the fiber, and that the entire wall of the fiber is involved in elongation. The present study further substantiates this view.

Summary

A technique involving the use of C^{14} in the study of cell wall formation in the cotton fiber is used to determine if the cotton fiber elongates only at the tip. After its introduction into the cotton plant in the form of carbon dioxide, C^{14} continues to be incorporated as cellulose in the walls of developing cotton fibers for a long period of time. After introduction of C^{14} into a cotton plant possessing partially elongated fibers, the entire cellulose framework of the primary wall becomes radioactive.

It is concluded that cell elongation in the cotton fiber involves cellulose synthesis throughout the length of the fiber wall instead of at the tip of the fiber alone.

The author wishes to express his appreciation to the University of Alabama Research Committee for a grant in aid which made this study possible.

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PHOSPHORYLASE AND A BRANCHING ENZYME IN SQUASH[1]

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In a study of the changes occurring in winter squash during storage (**10**) a large increase in sugar content was found to accompany the rapid disappearance of starch. As maltose was not found, it was thought that phosphorylase might be active in the breakdown of starch. Later TROMBLY (**13**) proved the presence of phosphorylase in the flesh of two varieties of *Cucurbita maxima* and in one of *C. moschata*. INOUE and ONODERA (**5**) have reported finding this enzyme in pumpkin.

To determine the distribution of the enzyme, five different fruits of Blue Hubbard squash were examined during four months of storage. The average activity of the flesh of these fruits was 30 units (Green and Stumpf) of phosphorylase in 100 gm. fresh weight. The fibrous material of the same samples, surrounding the seeds and connecting them with the flesh, had an average activity of 266 units in 100 gm. fresh weight. This fibrous material, then, is a relatively rich source of phosphorylase. It was of interest to attempt to prepare it free from other enzymes acting on starch, and to learn whether it is accompanied by a branching enzyme.

Material and methods

Medium sized fruits of *Cucurbita maxima* var. Blue Hubbard were used. The fiber was freed from seeds and weighed portions of it were kept frozen until they were extracted. To a portion of fiber in a Waring Blendor was added twice its weight of water. After thorough blending the mixture was centrifuged at high speed. The sediment was discarded, and the supernatant was adjusted with NH_4OH to pH 6.7 to 6.9.

Preliminary fractionations were made at 0.25 and 0.50 saturation by dissolving the calculated weight of $(NH_4)_2SO_4$ with the aid of a mechanical stirrer. The resulting mixture was immediately adjusted to pH 6.7 to 6.9 with NH_4OH. It was centrifuged after standing from 15 to 30 minutes. The sediments were suspended in suitable small volumes of 0.1 M citrate buffer at pH 6.8. A solution of $(NH_4)_2SO_4$ containing 50 gm. of the salt in 100 ml. of solution, adjusted to pH 6.8 to 6.9 with NH_4OH and filtered was used for further fractionations.

Phosphorylase activity was determined by a modification of the method of GREEN and STUMPF (**4**) and it is expressed in the units they proposed.

[1] Published with the permission of the Director as scientific contribution no. 153 of the New Hampshire Agricultural Experiment Station.

One unit liberates 0.1 mg. inorganic P in three minutes at 38° and pH 6.0. The digest had a final volume of 5 ml. It contained 1 ml. of 0.25 M glucose-1-phosphate dissolved in 0.1 M citrate buffer of pH 5.35, 1 ml. 0.75% Lintner starch and 1 ml. of the enzyme preparation suitably diluted with 0.1 M citrate buffer of pH 6.8. The final pH of the digest was 6.0. The vial containing the digest was placed in a water bath at 38°. When the digest had reached the temperature of the bath, a 1-ml. sample was removed for the determination of inorganic P by the method of FISKE and SUBBAROW (**3**). Removal of protein from the sample was not necessary. Exactly 30 minutes later inorganic P was determined in a second 1-ml. sample. From the increase in inorganic P, the activity of the enzyme was calculated in the units mentioned above. Under these conditions the liberation of inorganic P is a linear function of time over an extended period, hence its determination at zero time is not essential.

Qualitative tests for the presence of the branching factor or α-amylase were made by including 1 ml. of the enzyme preparation in a volume of 5 ml. which contained 0.2% Lintner starch and no added phosphate. Spot plate tests with I were made at intervals. If no change in the original blue color appeared in 20 hours, the preparation was considered free from branching factor and α-amylase.

Some of the preparations, protected with toluene, were dialyzed against several changes of distilled water for 24 hours. Solids were determined in aliquots of the resulting solutions by evaporation on the steam bath and completion of the drying in a vacuum oven at 65°. The protein content of 1-ml. portions of the same solutions was determined by the method described by LOWRY *et al.* (**7**).

One of the phosphorylase preparations which the above test indicated was free from α-amylase and the branching factor was used for the synthesis of polysaccharide from glucose-1-phosphate. The procedure was like that of BARKER *et al.* (**2**) but on a smaller scale. The 30 ml. of digest contained 0.1 gm. ester phosphorus as glucose-1-phosphate, and 17 units of phosphorylase. The original pH was 5.9. No primer was added. After 31 hours at room temperature approximately 80% conversion had occurred, and the pH was 6.05. Retrograded and nonretrograded starch were separated as described by the authors cited above.

For comparison with the synthetic starch, amylose was prepared from potato starch by the method of LANSKY *et al.* (**6**). The starch was autoclaved, and the amylose was precipitated with a mixture of equal parts of n-butyl and isoamyl alcohols. It was not refractionated. Blue values were determined by the method of MCCREADY and HASSID (**8**). As test tubes were used in the Klett instrument instead of 2-cm. cuvettes, the concentrations of starch, I and KI were doubled. The resulting figures are approximately 10% higher for comparable samples than those of the above authors. Iodine combining power was determined by the method used by LANSKY *et al.* (**6**).

Yields and the results of tests were calculated to 100% $C_6H_{10}O_5$ on the basis of analyses of the samples by the method of PIRT and WHELAN (**11**).

As all preparations of the supposed branching factor had some amylase activity, the following procedure was used to test for the presence of the branching enzyme. Digests were prepared containing 0.2% Lintner starch and suitable dilutions of the enzyme preparation in question and salivary amylase. Both were buffered with 0.1 M citrate to pH 6.8 and were kept at room temperature (22°). At intervals indicated by spot plate tests with I, samples were removed for the determination of blue value and reducing power as maltose by the iodimetric method of SOMOGYI (**12**).

Results

When the comminuted fiber was centrifuged, the supernatant was cloudy. Part of the suspended solids separated with each fraction. On suspending the fractions in citrate buffer, the insoluble solids flocculated and they were removed at the centrifuge. The first fractions contained 60 to 70% of the apparent phosphorylase activity of the original extract. The preparations resulting from the second fractionations contained from 25 to 35% of the original activity. The greater part of the phosphorylase activity appeared in the first fraction separated between 0.25 and 0.50 saturation with $(NH_4)_2SO_4$, some amylase and branching activity were present also. On further fractionation these contaminants were precipitated at 19 gm. $(NH_4)_2SO_4$ in 100 ml. of solution. The phosphorylase resulting from the third separation was usually free from them as shown by the test described above. No maltase was detected in these preparations.

In a 30-minute period of incubation the optimum temperature for the activity of the enzyme at pH 6.07 was between 40 and 45°. At 38° the optimum pH was between 6.0 and 6.1.

The apparent phosphorylase activity of the cruder preparations was inhibited 15 to 20% by 0.01 *N* NaF. The more refined preparations were not inhibited measurably by this addition. A phosphatase was present acting on β-glyceryl phosphate at pH 6.0, but none acting on glucose-1-phosphate.

The synthesis of polysaccharide, described above, resulted in the following yields in per cent. of the theoretical 80% conversion of the glucose-1-phosphate used: retrograded 51.3%, non-retrograded 29.2%, total yield 80.5% of the theoretical. The following results of tests were given respectively by amylose from potato starch, and the retrograded and non-retrograded synthetic polysaccharides: Blue value, 285, 324, 350; Iodine combining power, 17.65%, 20.25%, 20.10%.

In the dialyzed preparation, 1 mg. of solids corresponded to 0.80 unit of phosphorylase activity present in the non-dialyzed sample, and 1 mg. of protein to 1.05 units.

That portion of the squash fiber extract which, during fractionation, pre-

cipitated at 19 gm. $(NH_4)_2SO_4$ in 100 ml. of solution caused a rapid change of the color of iodine with starch to red. The red color gradually became paler and almost disappeared in 20 hours. It seemed probable that this fraction contained a branching enzyme, but also slight amylase activity. Comparison of its action on starch with that of salivary amylase was made as described above. The results of this experiment are presented in figure 1 in which blue values are plotted against milligrams of maltose liberated from 100 mg. starch. The time in minutes, from starting the digest until each sample was taken, is shown on the figure.

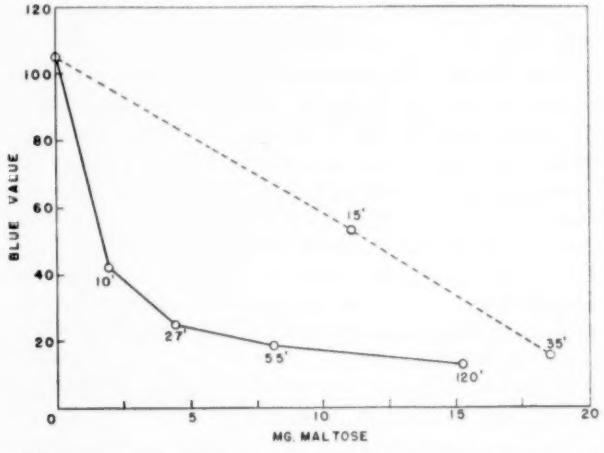

FIG. 1. Blue values and reducing power as milligrams of maltose resulting from the action on 100 mg. Lintner starch of the squash enzyme preparation (——) and salivary amylase (- - -). The time, in minutes from the addition of the enzyme to the taking of the sample is shown at each point.

Discussion

The phosphorylase of squash fiber can be freed easily from amylase and the branching enzyme. A preparation resulting from the third fractionation with $(NH_4)_2SO_4$ gave no qualitative test for those enzymes, and both the retrograded and non-retrograded starches formed by its action on glucose-1-phosphate gave blue values and iodine combining powers comparable with those of purified amyloses. Had either of the contaminating enzymes mentioned been present the non-retrograded starch would have shown evidence of partial breakdown.

As it was not the purpose of this study to obtain a highly purified preparation, no further fractionations were made. On the basis of activity per milligram of solids this preparation was slightly less than one third as active as those from potato reported by MEYER and DE TRAZ (**9**) and by GREEN and STUMPF (**4**). Its activity per milligram of protein was slightly more

than one third of that reported by the latter authors, 1.05 and 3.08 units respectively.

The branching enzyme was not obtained entirely free of amylase, but that it was present is shown by the results presented in figure 1. A true branching enzyme, like the Q enzyme of BARKER *et al.* (1), would develop little or no reducing power in starch, but by changing amylose to amylopectin it would decrease the blue value of the starch rapidly. If this enzyme were accompanied by small amounts of α-amylase, slow development of reducing power would result. Eventually, both reducing power and blue value would approach those values, developed in a shorter time, in a digest with α-amylase alone. As this was the result of the experiment described, it is concluded that a branching enzyme was present in the preparation.

Summary

Phosphorylase is abundant in the Blue Hubbard squash in the fiber that connects seeds with flesh. This enzyme can be prepared readily free from α-amylase and branching enzyme. A branching enzyme similar to the Q enzyme is present. This enzyme was not separated completely from α-amylase.

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THE EFFECTS OF MALEIC HYDRAZIDE ON FLOWER INITIATION

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Introduction

Since the biological effects of maleic hydrazide (MH) were first described by SCHOENE and HOFFMANN (**4**), a great many papers have appeared describing the effects of that compound on growth and flowering. NAYLOR (**3**) sprayed tobacco, maize and cocklebur with various concentrations ranging from .025% to .2% and obtained either suppression or delay of visible flowering. WHITE and KENNARD (**5**) delayed flowering in raspberries without any further deleterious effects on fruit set. FILLMORE (**1**) effectively inhibited vegetative growth and delayed flowering in *Vaccinium corymbosum* for two weeks. In an attempt to clarify the nature of the demonstrated inhibition of flowering by MH, the following experiments were conducted.

Experimental procedures

Plants whose flowering is sensitive to photoperiods were grown in subirrigated gravel beds under day lengths which prevent flower initiation. In the case of Wintex barley, a long-day variety, the plants were treated when they had developed to the point where the second leaf was mature. About one fourth of this second leaf was removed and the stump of the cut leaf immersed in a 10-milliliter vial containing an aqueous solution of the diethanolamine salt of MH. This method of treatment, as pointed out by LEOPOLD and THIMANN (**2**), not only insures the direct entrance of the solution into the plants, but allows more accurate control of the amount taken up by the plants. It permits use of low concentrations for a given effect; as for example, 4 mg. of MH per liter will be shown in the present study to prevent flowering entirely in Wintex barley when applied by the vial method. This concentration will not inhibit flowering to any detectable degree when sprayed on the plant.

Unless otherwise stated, short photoperiods consisted of nine hours, and long photoperiods consisted of 18 hours. Normal daylight was extended with reflector flood lamps of 150 watts furnishing between 100 and 125 footcandles of light at the leaf surface. Each treatment was applied to 10 plants selected at the beginning of the treatment for uniformity.

In the first series of experiments barley plants were treated with the following concentrations of MH: 0, 4, 10, 40, 100, and 200 milligrams per

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liter. The plants were treated for five days, and during treatment they were exposed to long photoperiods in order to induce flowering. The vials were then removed, and plants were returned to short photoperiods. After two weeks the plants were dissected and the number of flower primordia, fresh weights, and number of tillers recorded. Table I shows the results of a typical series of these treatments.

Maleic hydrazide in concentrations as low as 4 mg. per liter was found to prevent the formation of flower primordia in barley. Some plants after treatment with concentrations of 50 mg. per liter died, and all plants died when treated with concentrations of 100 or 200 mg. per liter. The fresh weight of the plants decreased with increasing concentrations of MH, and tillering was increased by the low concentrations of MH (table I). These results can be reproduced readily as long as plants at the same stage of development are used. Older plants are less responsive.

TABLE I

EFFECT OF MALEIC HYDRAZIDE ON NUMBER OF FLOWER PRIMORDIA, FRESH WEIGHT, AND NUMBER OF TILLERS IN WINTEX BARLEY (AVERAGES OF 10 PLANTS PER TREATMENT).

Concentration	Fresh weight	Tillers per plant	Flower primordia per plant
mg./l.			
0	1.8	0.1	13.0
4	1.14	2.3	0.0
10	1.10	1.2	0.0
20	0.95	1.4	0.0
50	0.67	0.4	0.0
100	*	*	*
200	*	*	*

* Dead as result of treatment.

It seems that here is an instance of the complete prevention of photoperiodic flower initiation by apparently nontoxic quantities of a chemical which is not a growth regulator. Under conditions reported, the treatment with 4 mg. per liter completely prevents flower initiation without causing any visible deformities or outward symptoms of toxicity.

In order to determine whether similar effective control could be shown for plants with different flowering habits, experiments were carried out with Biloxi soybean, a short-day plant. The plants were grown for four weeks under long photoperiods and then treated under short photoperiods. The youngest mature leaf (the third trifoliate leaf) was cut and immersed as described for barley. The period of treatment was extended to seven days. The plants were dissected four weeks after commencement of treatment.

As is shown in figure 1, the number of primordia appearing on the first five nodes above the treated leaf decreased as the concentration of MH was increased. However, flower initiation was less inhibited than in the case of

barley. Treatment with 4 mg. per liter resulted here in only 10% reduction in the number of flower primordia. At concentrations of 40 and 100 mg./l. the apices died. The lateral shoots had no flower primordia when dissected. Flower initiation in the apical buds was not completely inhibited by MH unless the apical bud was killed by the treatment. It seems evident from the data of this experiment and other repetitions of the same that flower

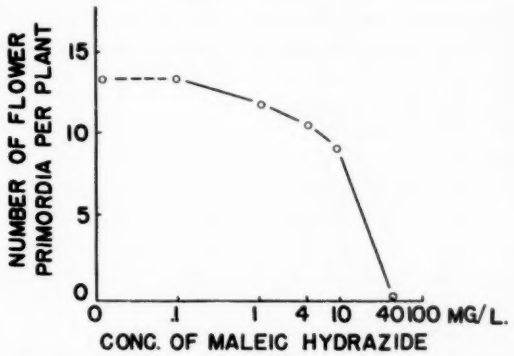

FIG. 1. The effect of maleic hydrazide on the total number of flower primordia on the first five nodes in Biloxi soybean.

initiation in soybean cannot be completely controlled with MH, as it can in barley.

In order to secure more evidence on MH effects on flowering, tests were carried out on Marketeer chrysanthemum and peppermint. Marketeer chrysanthemum is a short-day plant, and peppermint is a long-day plant.

Chrysanthemum cuttings were rooted during the month of May, transplanted into gravel beds, and grown under long photoperiods until September. They were then subjected to a foliar spray with six concentrations of MH ranging from 0 to 5000 mg./l. just prior to the commencement of short photoperiods. The sprays were applied early in September, 1950, using 18

TABLE II

EFFECT OF MALEIC HYDRAZIDE ON THE NUMBER OF TERMINAL FLOWERS ON MARKETEER CHRYSANTHEMUMS (AVERAGES OF 18 PLANTS EACH).

Concentration	Flowers November 27, 1951	Flowers December 15, 1951
mg./l.		
0	2.3	2.4
100	1.6	2.0
500	0.3	0.3
1000	0.0	0.0
2500	0.0	0.0
5000	0.0	0.0

plants per treatment, after which the plants were subjected to the natural short days until harvest. The number of visible terminal flowers were recorded at intervals as shown in table II. The formation of terminal flowers was effectively inhibited with MH at concentrations of 500 mg./l. and higher. However, lateral flowers were produced on all plants which were allowed to develop lateral shoots, regardless of treatment with MH.

Peppermint plants were grown to a height of about six inches in flats of peat soil. At this stage they were given a foliar spray with 0, 500, and 2000 mg./l. of MH and exposed to 18-hour photoperiods. The apices of the

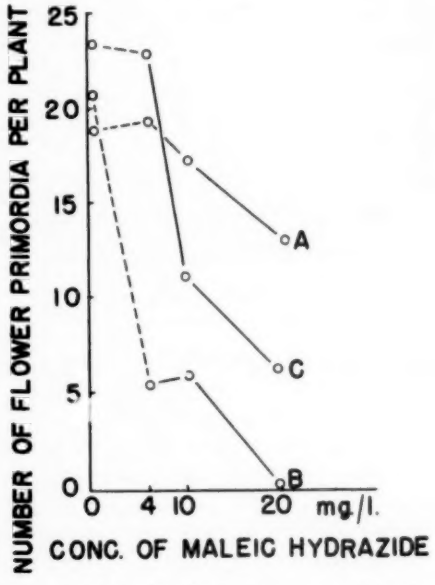

FIG. 2. The effect of maleic hydrazide on the number of flower primordia in Wintex barley. A. Apex removed at end of treatment with MH. B. Apex removed at commencement of treatment with MH. C. Apex left intact.

shoots sprayed with MH were totally inhibited in growth and failed to resume growth even after four months. However, the lateral buds grew and produced flowers on all treatments.

The fact that chrysanthemum and peppermint produced flowers on lateral shoots even though the plants had received applications of high concentrations of MH suggests that this inhibitor may be acting against the terminal growth and not specifically against flower initiation. In an attempt to distinguish between the effects of MH on growth and on photoperiodic induction, the following experiments were conducted: After Wintex barley plants had been grown for 23 days, MH was applied at concentrations of 0, 4, 10,

and 20 mg./l. Three such sets of treatments were used. One set of plants had the apices removed with a needle immediately before beginning treatment with MH; another set had the apices removed immediately after treatment with MH (five days); and a third set did not have the apices removed at all. The removal of the apices was carried out in order to force the development of lateral buds or tillers. The MH was applied for five days, but the photo-induction period was extended to 12 cycles. The number of flower primordia on the apex or on the most vigorous tiller which replaced it were counted two weeks after the treatment was started.

As can be seen in figure 2, the laterals forced at the beginning of treatment (curve B) were completely prevented from forming any flower primordia by 20 mg./l. of MH and the number formed was greatly reduced by lower concentrations. In contrast, the laterals forced after treatment (curve A) were very much less affected by the MH. The number of flower primordia was only reduced 44% by the highest concentration. The plants which did not have the apices removed at all (curve C) were not completely prevented from forming primordia, but the number was reduced by 71% at the highest concentration.

The same experiment was repeated on plants which were seven days younger than the above plants. The results were similar, except that on the younger plants MH was considerably more effective.

Discussion

The present experiments have shown that MH will prevent the formation of flower primordia in Wintex barley at concentrations as low as 4 mg./l. The other plants tested show a lesser inhibition of flowering by MH. When flowering is prevented in apical buds, laterals can frequently develop flowers.

If MH acts specifically against the flowering stimulus, it should be just as effective after a given period of induction as at the beginning. Thus, lateral buds forced into development after five days of induction should show no less inhibition of flowering than those forced at the beginning of treatment. If, on the other hand, MH acts only against growth of active apical meristems, then lateral buds forced into development after the MH treatment had been stopped might be expected to show less inhibition of development than those forced at the beginning of treatment. In the last experiment all plants received the same photoperiodic induction and the same duration of MH treatments. Under these conditions the flowering stimulus should be equally affected by a flowering inhibitor, regardless of time of growth. This is not the case, so MH cannot be considered to be a true flowering inhibitor.

The apparent action of MH against the formation of flower primordia seems to be through its effective inhibition of growth, rather than by any specific action against the photoperiod mechanism itself. Where terminal growth is retarded by MH, flowering in that terminal meristem is either

inhibited, reduced or delayed. However, where growth is not retarded (*e.g.*, in lateral branches appearing after MH treatment) flower primordia are produced, even though the plant has been treated with MH.

Summary

Maleic hydrazide was found to inhibit completely the formation of flower primordia in Wintex barley, a long-day plant, at concentrations as low as 4×10^{-5} M. The effective inhibitory concentration of maleic hydrazide was found to vary with the age and size of the plant. Photoperiodic induction of Biloxi soybean, a short-day plant, was inhibited somewhat by maleic hydrazide but the treatment did not completely suppress floral initiation. The apex died back after treatment with approximately 40×10^{-5} M maleic hydrazide. Foliar application of maleic hydrazide on Chrysanthemum and peppermint inhibited flowering in terminal buds, but not in laterals.

Evidence was brought forth suggesting that maleic hydrazide inhibits the production of flower primordia primarily through its inhibitory effect on growth, rather than by any specific action against the photoperiodic mechanism itself.

The authors wish to express appreciation to the Naugatuck Chemical Company for furnishing the maleic hydrazide with which these experiments were conducted.

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PREFERENTIAL ASSIMILATION OF AMMONIUM ION BY *CHLORELLA VULGARIS*

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Introduction

It is generally known that, when the culture medium contains ammoniacal nitrogen as well as nitrate and nitrite nitrogen, green algae utilize the ammoniacal nitrogen. However, when the supply of ammoniacal nitrogen is limited, there is a question as to the selectivity of the organism with regard to the nitrogen available. This situation arises when algal cells deplete the medium of the ammoniacal nitrogen originally supplied and are forced to rely on nitrogen in other forms, including those compounds arising from the metabolic activities of the cells.

A series of experiments has been performed by the authors as a part of a general program concerning the nitrogen utilization of algae, using heavy nitrogen as a tracer and the mass spectrometer as an instrument for its assay. Like many experiments in which the newer techniques have been used, the results are not unusual but merely confirm, by a relatively independent technique, what has already been known. For example, PRATT and FONG (**7**) showed that cultures of Chlorella preferentially utilize ammonium as long as it is present, and then absorb nitrate. An advantage of the method employing a tracer and the mass spectrometer is that it is possible to get data concerning the source of the cell nitrogen when the nitrogen is presented to the organism as both ammoniacal and nitrate nitrogen simultaneously. No precautions were taken to prevent the algae from fixing atmospheric nitrogen during the experiments.

Methods and materials

The medium for the growth of the alga consisted of inorganic salts and glucose. The only source of nitrogen in the medium was ammonium nitrate. Five liters of medium containing 0.20 gm. NH_4NO_3, 0.08 gm. KH_2PO_4, 0.08 gm. $MgSO_4 \cdot 7\,H_2O$, 0.04 gm. $CaCl_2 \cdot 2\,H_2O$, 0.4 mg. $FeSO_4 \cdot 7\,H_2O$, and 20.0 gm. glucose per liter were prepared with distilled water. The ammonium nitrate used had been obtained from Eastman Kodak Co. and the specified atom per cent. of N^{15} in the ammonium radical was 61.5. A determination of this value using the atomic masses for the ratio 15/14 was 61.1 atom per cent. which was accepted as the standard for all of the other ratio measurements. For convenience in handling, the five liters of medium were divided

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into two portions of 2500 ml. and each portion put into a four-liter serum bottle. The bottles were plugged and autoclaved at 15 pounds for 30 minutes.

A pure culture of *Chlorella vulgaris* Beijerinck was used for inoculation. This organism had been isolated as a unialgal culture free of bacteria and fungi in this laboratory, and had been identified by Dr. Francis Drouet of the Chicago Natural History Museum. The inoculum culture was made by putting a loop of the cells from a slant culture into a tube containing 15 ml. of liquid medium of the composition described previously, except that non-labeled NH_4NO_3 was used. The culture was incubated at 21° C for seven days under white fluorescent lamps which provided an illumination of 250 foot-candles. Five milliliters of this inoculum culture were used for inoculation of each of the two serum bottles.

After seven days of growth under the temperature and light conditions listed above, the bottles were removed from the incubator, the cells centrifuged from the medium, washed twice with distilled water, and then frozen. The medium was resterilized in two four-liter serum bottles and reinoculated. The same process of removing cells, resterilizing medium, and reinoculation was repeated twice again, giving in all, four experiments. The time of growth for each of these was 7, 6, 10, and 16 days respectively.

Analyses for ammoniacal nitrogen were made on the media, and the cells were analyzed for total nitrogen. Total nitrogen determinations were done by the Kjeldahl method, modified to include the nitrogen of nitrates (**1**). The ammoniacal nitrogen determinations on the media were done by means of an alkaline distillation into standardized HCl solutions.

Samples for the mass spectrometer were prepared by the same methods except that the excess standard acid into which the NH_4 had distilled was not titrated with standard NaOH. The contents of the receiving flask were boiled down, and then the ammonium oxidized to molecular nitrogen by means of the hypobromite method and apparatus suggested by RITTENBERG (**8**).

A Nier-type mass spectrometer (**2, 3, 4, 5, 10**) was used to measure the relative abundance of atomic nitrogen, defined as

$$\frac{N^{15}}{N^{14} + N^{15}} \times 100 = \text{atom per cent. of } N^{15}.$$

The atomic masses (N^{15}/N^{14}) were chosen in preference to the conventional molecular masses (N^{29}/N^{28}) because this procedure gives freedom from interference by residual carbon monoxide, better spectral separation for smaller masses, and quicker and more simple computation of results since molecular combinations of N^{14} and N^{15} involve additional statistical calculations. Since the change in the abundance ratio was of primary concern, all ratio measurements were compared with the standard sample. Thus any isotope discrimination which occurs during dissociation at the ion source is eliminated in the relative abundance ratios. The instrument used was constructed (**9**) in the Physics Department of the University of Cincinnati

TABLE I

MASS SPECTROMETRIC DATA CONCERNING NITROGEN METABOLISM IN *Chlorella vulgaris*. THE AVERAGE VALUES SHOWN ARE RELATIVE TO THE ATOM PER CENT. OF N^{15} IN THE AMMONIUM RADICAL OF NH_4NO_3 IN THE CULTURE MEDIUM AT THE BEGINNING OF EXPERIMENT 1 (61.1).

Experiment	Age of medium	Material	Atom per cent. N^{15} in total N
	days		
1	0	Medium at start of experiment	30.5
		Cells at end of experiment (7 days)	55.5
2	7	Medium at start of experiment	24.4
		Cells at end of experiment (6 days)	55.4
3	13	Medium at start of experiment	18.1
		Cells at end of experiment (10 days)	13.9
4	23	Medium at start of experiment	7.9
		Cells at end of experiment (16 days)	5.4
	39	Medium at end of all experiments	8.0

essentially according to the description of NIER (**3**). The magnetic analyzer and the FP-54 electrometer circuit are patterned fundamentally after those described earlier by NIER (**4, 6**). From a sample of ammonium chloride containing approximately 1.5 mg. of nitrogen, obtained from the Kjeldahl process and oxidized by a hypobromite solution (**8**), sufficient molecular nitrogen was obtained to fill a 15 ml. bulb at a pressure of 10 cm. Hg. This sample was introduced into the mass spectrometer with the technique used by NIER (**3**).

Results and discussion

The original medium contained equal proportions of ammoniacal and nitrate nitrogen as NH_4NO_3 in a concentration of 0.2 mg./ml., and no other form of nitrogen. In this environment the algal cells appear to have had a surplus of NH_4 available at the beginning of their growth. In consequence, the cells harvested at the end of the first experiment (seven days) had 55.5 atom per cent. of N^{15} and the resulting medium had 24.4 atom per

TABLE II

CHEMICAL DATA CONCERNING NITROGEN METABOLISM IN *Chlorella vulgaris*. ALIQUOTS OF THE SAME MEDIUM FOR WHICH THE MASS SPECTROMETRIC ANALYSES APPEAR IN TABLE I WERE USED FOR THE CHEMICAL DETERMINATIONS.

Experiment	Age of medium	Ammoniacal nitrogen	pH
	days	*μg./ml.*	
1	0	35	4.6
2	7	23	3.2
3	13	6	3.0
4	23	5	3.3
	39	3	3.5

cent. of N^{15}. These figures show that the cells definitely preferred NH_4 to NO_3. However, the value of 55.5 atom per cent. of N^{15} (instead of 61.1 atom per cent. of N^{15}) in the harvested cells, seems to indicate that even with a supply of NH_4 available, the cells were utilizing a form of nitrogen other than NH_4. A situation similar to that of experiment 1 is present in experiment 2, except that, by the end of the latter, the medium has been depleted of NH_4 due to preference for it over all other forms of nitrogen. This depletion is reflected in the 55.4 atom per cent. of N^{15} in the harvested cells.

At the beginning of experiment 3, practically no NH_4 was available in the medium and so the cells grew according to a new selection of nitrogen from additional sources other than the labeled ammoniacal nitrogen. The medium now contains nitrogen compounds of N^{15} as by-products of cell growth and decay. The cells contain the atom per cent. of the medium, but apparently do so according to two different mathematical factors. At the very beginning of the first experiment the only N^{15} is in the NH_4 radical of the NH_4NO_3 in the medium. Therefore the total nitrogen in the medium has an atom per cent. of N^{15} equal to one half that of the ammonium radical. When the cells take up NH_4 predominately, their atom per cent. should, and did, equal approximately twice that of the medium. However, when N^{15} in the medium is in compounds other than ammonium (presumably resulting from algal metabolism) and when, at the same time, very little NH_4 is available, the cells have an atom per cent. of N^{15} approximately equal to that of the medium.

The fourth experiment seems to be an extension of the third. The atom per cent. of N^{15} in the starting medium, in the final medium, and in the harvested cells was approximately the same which indicates that a type of dynamic equilibrium has been reached, in that the cells are removing representative nitrogen samples from the medium (removing N^{14} and N^{15} in the same proportion as these are found in the medium) and returning the same proportions to the medium in metabolic products.

Summary

A pure culture of *Chlorella vulgaris* was grown in an organic medium containing NH_4NO_3 having 61.1 atom per cent. of N^{15} in the ammonium radical. After a period of growth, the cells were harvested and the cell-free medium resterilized and reinoculated. This process was repeated three more times. At the end of each experiment, analyses for N^{15} in the medium and cells were performed using a mass spectrometer.

It was found that the algal cells definitely preferred ammoniacal nitrogen to the nitrate form since their atom per cent. of N^{15} approximated twice that of the medium. However there was evidence that nitrogen from a source other than the ammonium supplied in the medium was retained by the cells even when a plentiful supply of ammoniacal nitrogen was present. When practically no NH_4 was available in the medium, the cells grew

according to a new selection of nitrogen from sources other than the labeled ammoniacal nitrogen. The medium now presumably contained nitrogen compounds of N^{15} as by-products of cell growth and decay. It was found that the cells had an atom per cent. of N^{15} which equalled that of the medium.

This study has been made possible by a stipend for a post-doctoral fellowship in Biophysics at the University of Cincinnati (John F. Schuler) established by the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and by grants for equipment, supplies, etc., from the Research Corporation, New York City (1949–51) and from the National Institutes of Health, Bethesda, Maryland (1951–52).

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RESERVE CARBOHYDRATES IN ORCHARD GRASS CUT FOR HAY[1]

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Cyclic changes in some of the constituents of orchard grass clipped every 35 days were described in a previous paper (**1**). Coincident with that experiment, another group of plants was allowed to reach a more mature growth, an early hay stage, and then clipped. The composition of these plants, both before and after clipping, is reported here. The objectives of this experiment were similar to those of the other one, namely, to determine the fluctuations of carbohydrate reserves in the stubble and roots after the tops were cut and during a recovery period, and to note the effects of nitrogenous fertilization on those changes.

The orchard grass was of the same clone and was subjected to similar cultural treatment and to the same methods of analysis as in the work previously reported, with the exceptions described below. It was first grown under the natural day length of late fall (about 9.5 hours), and was then placed under a day length of 16 hours, conditions which led to heading. It was estimated that an early hay stage would be reached on February 2, 1944, about a week before flowering. This day was therefore chosen as day 0 (zero) and the plants were divided into six series, five to receive the same amount of nitrogenous fertilizer, but at different times according to the following schedule: Series A, no nitrogen, series B, nitrogen on January 19 (day – 14), series C, nitrogen on January 26 (day – 7), series D, nitrogen on February 2 (day 0), series E, nitrogen on February 9 (day 7), and series F, nitrogen on February 16 (day 14). The nitrogenous fertilizer consisted of 0.4 gram of urea per pot, a rate equivalent to about 100 pounds of nitrogen per acre. In addition, monopotassium phosphate, at a rate of 200 pounds per acre, was added to all pots on day – 14.

Some of the plants were taken up for chemical analysis on each of the days indicated in the figures. By day 0 they had headed but not flowered; they were considered to be at the hay stage and all plants were cut. The aftermath of subsequent dates was composed entirely of leaves. Plants which received nitrogen were not sampled until the next sampling date after the nitrogen application, and were considered up to that time as part of series A. The experiment was conducted in duplicate, and the plants from two separate greenhouse benches were sampled and analyzed separately and all data are reported as the averages of the two replications.

[1] Contribution no. 116 of the U. S. Regional Pasture Research Laboratory.

Results

Dry matter yield

Top growth was, in general, increased by nitrogen fertilization (fig. 1). The fertilizer applied on day – 14, when the reproductive parts of the plants were well developed, appeared to have increased the yield of series B slightly, but not significantly, by the time of cutting, but that applied on day – 7 when the plants were in full head had no effect on yield.

In the aftermath harvested after day 0, differences in yield between the series were evident. All plants that received nitrogen produced more top

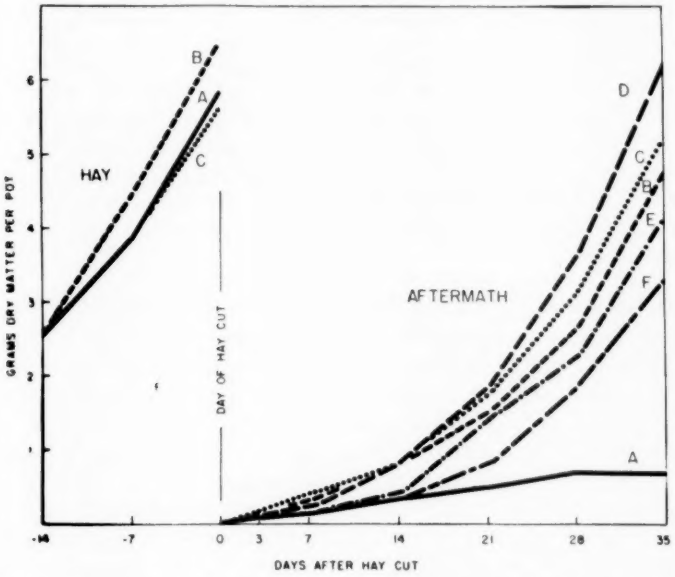

Fig. 1. Changes in the dry weight of the top growth of orchard grass, before and after hay cut, in grams per pot. Letters indicate time of application of nitrogen; A, no nitrogen; B, nitrogen applied on day – 14, C on day – 7, D on day 0, E on day 7, and F on day 14.

growth than series A. The highest yield obtained was from series D which was fertilized on day 0 and the lowest, except for A, were from E and F which were fertilized 7 and 14 days, respectively, after day 0. Those fertilized before day 0 when the plants were making their hay growth were intermediate in yield of aftermath. Significance was calculated from the data of day 35 from the fresh weights of tops from the six pots in each series which were weighed before they were dropped into alcohol. Significance existed among all series except between B and C.

The stubble and roots underwent losses in dry weight after the hay was cut and after nitrogen fertilizer was applied (table I). Low points were

TABLE I

DRY WEIGHTS OF STUBBLE AND ROOTS OF ORCHARD GRASS, AS GRAMS PER POT, AT THE DAY WHEN CUT FOR HAY AND AT SEVEN AND 35 DAYS THEREAFTER. FIGURES IN PARENTHESES RELATE TO PLANTS NOT YET FERTILIZED: HENCE THEY ARE THE SAME AS SERIES A UP TO THAT TIME.

Series	Day when nitrogen was added	Weight of stubble			Weight of roots		
		Day 0	Day 7	Day 35	Day 0	Day 7	Day 35
A	No nitrogen added	3.07	2.60	2.84	2.20	2.03	3.81
B	Day −14	3.40	2.61	3.60	2.02	1.74	3.30
C	Day −7	3.19	2.80	4.00	2.19	1.94	3.56
D	Day 0	(3.07)	2.72	4.40	(2.20)	1.95	3.29
E	Day 7	(3.07)	(2.60)	3.81	(2.20)	(2.03)	3.30
F	Day 14	(3.07)	(2.60)	3.90	(2.20)	(2.03)	3.20

reached at about day 7, but by day 35 the weights had again increased. The stubble of series A, which did not receive nitrogenous fertilizer at any time, did not return to its weight of day 0, but the stubble of all other series exceeded their weights of that date. On the other hand the roots of the unfertilized series A were greater in weight than the roots of any of the fertilized series.

CARBOHYDRATE CONTENT OF THE STUBBLE AND ROOTS

Percentages of fructosan and sucrose in the stubble are presented in graphic form in figure 2. Fructosan was the dominant water-soluble carbohydrate, and in the unfertilized plants attained 26% of the dry weight on day 35. There were no decreases in the fructosan of the stubble as an immediate result of removing the tops on day 0, but there were decreases in each of the fertilized series soon after nitrogen was applied. These decreases were not great in some of the fertilized series, but were relatively great when compared with the increasing amounts of fructosan in the unfertilized series A. Sucrose on the other hand showed typical u-shaped curves of utilization and restorage after day 0, with a low point in all series about 21 days after cutting.

In the roots, the changes in fructosan and sucrose were similar to those in the stubble but, in quantity, fructosan was relatively lower and sucrose relatively higher (fig. 3). In series A, fructosan rose from 7% at time of hay cutting on day 0 to 13.4% on day 35. There were no consistent decreases in fructosan as a result of cutting at the hay stage, but there were decreases in each of the fertilized series when nitrogen was applied. Sucrose on the other hand showed the typical u-shaped curve after the cutting with a low point on day 21 and also decreased as a result of fertilization. Reducing sugars were lower, amounting to 2.6% or less in the stubble and to 1.0% or less in the roots. Their quantitative changes were similar to those of sucrose, with a low point occurring two to four weeks after the cutting. Unlike sucrose, however, reducing sugars were higher in the fertilized than in the unfertilized plants, a result noted elsewhere (1).

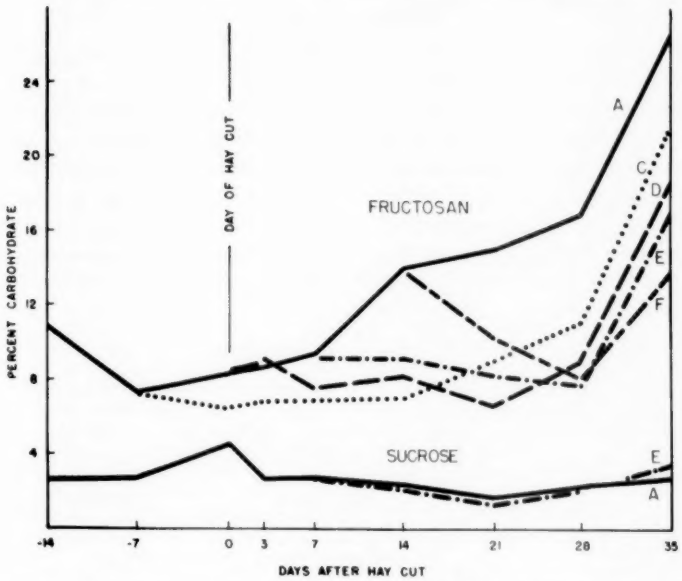

FIG. 2. Changes of fructosan and sucrose in the stubble of orchard grass, in percentage of the dry weight. Letters indicate treatment as in figure 1.

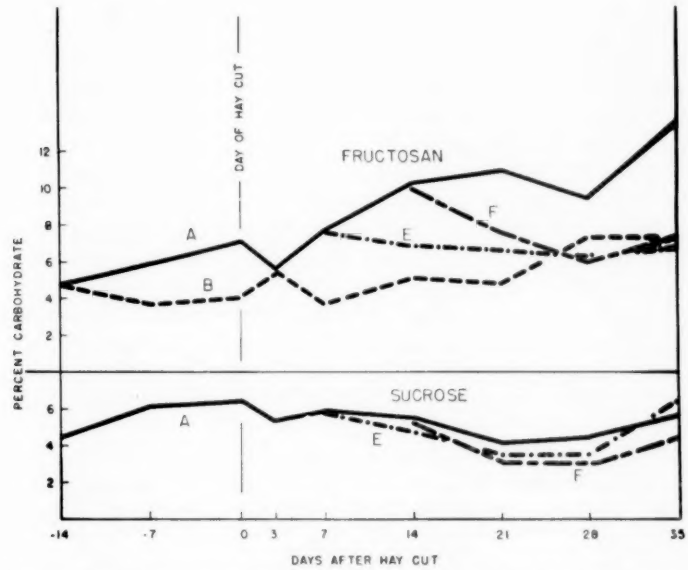

FIG. 3. Changes of fructosan and sucrose in the roots of orchard grass, in percentage of the dry weight. Letters indicate treatment as in figure 1.

Nitrogen content

The hay from series A contained 2.38% total nitrogen of which 13% was in the alcohol-soluble fraction (table II). While the addition of nitrogenous fertilizer to series B and C before the cut increased their nitrogen contents to 3.17 and 3.02% respectively, their yields were not increased appreciably. In the aftermath, nitrogen percentages were highest when the fertilizer was applied nearest to the time of harvest as illustrated in series E, and especially in series F, for the cuts made on day 35. These late applications also caused the smallest increase in dry matter (fig. 1). The proportions of the total nitrogen found in the alcohol-soluble fractions were not related to the fertilizer treatments.

TABLE II

TOTAL AND SOLUBLE NITROGEN CONTENTS OF ORCHARD GRASS HAY AND OF AFTERMATH 35 DAYS LATER. TOTAL NITROGEN IS IN PERCENTAGE OF THE DRY WEIGHT AND SOLUBLE NITROGEN IN PERCENTAGE OF THE TOTAL NITROGEN.

Series	Day when nitrogen was added	Hay on day 0		Aftermath on day 35	
		Total nitrogen	Soluble nitrogen	Total nitrogen	Soluble nitrogen
A	No nitrogen added	2.38	13.0	2.43	13.0
B	Day −14	3.17	18.2	2.28	12.6
C	Day −7	3.02	18.2	2.29	13.3
D	Day 0	....	....	2.46	11.4
E	Day 7	....	....	3.04	14.4
F	Day 14	....	....	3.72	11.3

In the stubble, total nitrogen increased temporarily after cutting the hay on day 0 and also increased, at least relatively, in each series after fertilizer application (fig. 4). The increases that followed the application of fertilizer occurred in both soluble and insoluble fractions, but were greater in the soluble, thus changing the ratios of the soluble to total nitrogen (fig. 5). Higher ratios were found to some extent after defoliation and especially after fertilization, but the differences in these ratios among series tended to disappear in time.

In the roots, the total nitrogen and insoluble nitrogen were less than in the corresponding stubble but the soluble nitrogen was frequently as great or greater. A graphic representation resembles that of the stubble but indicates more decided increases in the proportion of soluble nitrogen following fertilization.

Qualitative tests for nitrates were negative in series A but were positive in each of the fertilized series on the first sampling date following fertilization and they remained positive for three to six weeks in all parts of the plants.

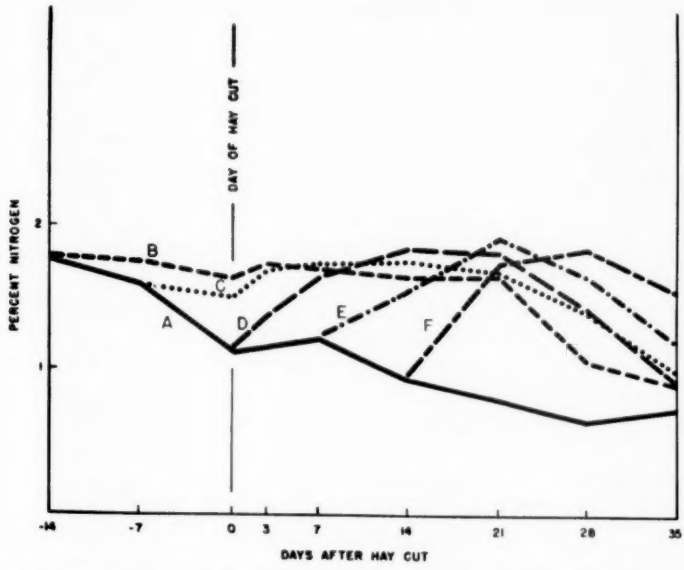

FIG. 4. Changes in the total nitrogen of the stubble of orchard grass, in percentage of the dry weight. Letters indicate treatment as in figure 1.

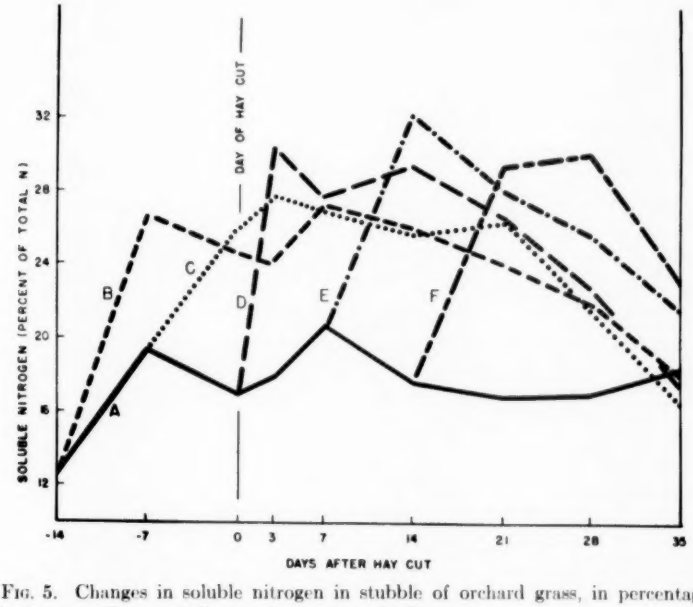

FIG. 5. Changes in soluble nitrogen in stubble of orchard grass, in percentage of total nitrogen. Letters indicate treatments as in figure 1.

Discussion

The compositional changes described here as having taken place in the stubble and roots of orchard grass agree in general with those found previously. The findings of this and three previous reports (**1, 2, 3**) may be summarized briefly. The study of reserves falls into three possible phases: (a) conditions underlying the accumulation of reserve carbohydrates, (b) the course of the utilization and restorage of carbohydrates after plant defoliation, and (c) the relations of reserve carbohydrates to plant behavior, such as production and survival. The reports cited have been concerned primarily with the second of these phases.

Reserve substances are considered to be those organic materials stored at certain times in the plant and utilized later as a source of energy or as structural material. In perennial rye grass and orchard grass, sugars and fructosan appeared to comply with this classification in that their concentrations underwent marked fluctuations following defoliation and during the recovery after defoliation. The structural substances, cellulose and hemicellulose, did not undergo fluctuations to such a degree as did the more soluble carbohydrates and they have not been studied further. The greatest accumulation of the soluble carbohydrates occurred in the lower parts of the leaf blades and sheaths, a combination of parts referred to here as the stubble, as a net result of negative downward gradient of sugars and a positive downward gradient of fructosan, the latter being most abundant. Of the sugars, sucrose alone displayed a gradient; that for reducing sugars was not definitely demonstrated. In the roots total carbohydrates were lower than in the stubble on a percentage basis, but on an absolute basis were sometimes larger. Fructosan was usually the predominant reserve carbohydrate of the stubble, and sucrose was often the predominant carbohydrate of the roots.

When the tops were cut, a rapid decrease of the stored carbohydrates occurred within three days, and probably much earlier since new leaf growth was often evident within a few hours after the cutting. It is possible that, because of the lowered photosynthetic area on clipped plants, carbonaceous matter for the new leaf tissue, produced during the first few days after cutting, was obtained primarily from the reserves. While this conversion of reserves into organic structural materials is not definitely proven, it is strongly suggested by the fact that new leaves were produced in almost equal amounts in both light and darkness during the first four days after defoliation. It is also probable that, after four days of recovery, the major quantity of carbonaceous matter lost from the stubble and roots was utilized in respiration rather than in increasing the dry weight of new tissue. In most and perhaps all cases the weight lost from the stubble and roots was considerably more than the weight of new tissues formed even under favorable growing conditions. In darkness this loss of weight over a period of 36 days was 4.26 grams, of which 1.21 grams was soluble carbohydrate,

while at the same time the weight of the new leaves was only 0.83 gram (**2**). The difference is assumed to be largely that lost by respiration. When plants were allowed to recover from clipping at different temperatures in light, the amount of new growth after 40 days was not in proportion to the amount of carbohydrate utilized (**3**). At the highest temperature, 80 to 90° F, at which respiration was more rapid, new leaf growth amounted to only 0.45 gram in nine days, and to only 0.77 gram in 40 days, while the weight losses from the lower plant parts were 2.31 grams and 7.16 grams, respectively, for the two dates. Even at a more favorable growth temperature, 60 to 70° F, the new leaf weight was 1.69 at 40 days and the weight loss in the stubble and roots was 2.84 grams. It seems therefore that a major amount of the carbohydrate reserve disappearing from the lower parts of plants after defoliation entered into the process of respiration. The new growth would depend for its raw material partly on reserves and partly on new photosynthetic products.

The relation between photosynthetic area and the accumulation and utilization of reserves needs clarifying. In the temperature studies just referred to, restorage of carbohydrates did not occur at the highest temperature, a fact in accordance with a high respiratory loss and a low photosynthetic area. In another experiment plants were grown at high nitrogen levels in contrast with those at low. The high nitrogen plants produced larger top growth and more photosynthetic area but on a percentage basis this did not result in greater carbohydrate storage within the limited time of the experiment. The high nitrogen plants also had a small weight of roots, but it is probable that if the experiment had run longer the increased leaf area would make possible a greater development of the root system and a greater carbohydrate storage in the underground parts. After clipping, the rate of utilization of the stored carbohydrate was similar in both high-nitrogen and low-nitrogen plants but the addition of nitrogen to plants without cutting also promoted an immediate utilization of stored carbohydrates. Thus, either the cutting of the tops or the addition of nitrogen stimulated top growth and each of these actions promoted utilization of reserve carbohydrates. It is a matter of observation that aftermath growth is delayed if the grass has been cut at the hay stage rather than at a more vegetative stage even though sufficient reserve carbohydrate may be present in each case. This delay may have a morphological basis in that there are fewer growing buds on the crowns of the grass at the hay stage, but there may also be a retarded development of these buds because of the lower photosynthetic area remaining after a hay cut. It is also to be noted in passing that more fructosan was utilized when the grass was cut at the hay stage than when it was cut at a more immature stage, and sucrose was used up rapidly without respect to the stage of maturity. Recovery after defoliation, as measured by new leaf growth, was more rapid when some photosynthetic area was left intact.

Disturbances of the nitrogenous compounds as a result of defoliation were as great as those of carbohydrates. The soluble nitrogen of the stubble

and roots increased quickly after the cutting of the tops, moderately if no nitrogen was applied, but greatly so if nitrogenous fertilizer was applied at the time of cutting. With or without cutting, nitrogenous fertilizer increased soluble nitrogen as well as total nitrogen. The soluble nitrogen increases were mainly due to increased absorption from the soil, but when defoliation occurred without concomitant fertilization, the increased soluble nitrogen originated either from protein hydrolysis or from an increased rate of nitrogen absorption by roots. Either or both processes would explain the nitrogenous fluctuations.

Relation of certain factors to aftermath yield

An experiment was conducted to determine whether any relation exists between the amount of stored carbohydrate in a plant and its recovery, as measured by top growth, after clipping. Since this experiment was preliminary in nature its description will be brief. A range of carbohydrate levels was produced in orchard grass by varying the environmental conditions of temperature and light and by varying the frequency of clipping. There were 10 series, ranging in weight of stubble from 1.1 to 3.2 grams (dry matter per pot) and of roots from 0.45 to 2.0 grams. The total soluble carbohydrates ranged from 6.3 to 22.3% of the stubble and from 1.3 to 10.3% of the roots. They were clipped uniformly and placed together for recovery. The aftermaths 25 days and 40 days later were compared to the weights and composition above. At each sampling four pots from each series were combined. There were no further replications, and it is admitted that statistical treatment was inadequate but correlation coefficients were calculated. Significant r values were obtained between yields of aftermath on the twenty-fifth day as one variable and certain data taken at the time of uniform clipping as the other, such as the dry weight of the stubble (+0.85, significant at 1% point), the dry weight of the roots (+0.80, significant at 1% point), and the dry weight of the stubble and roots combined (+0.83, significant at 1% point) (D.F. = 8). A significant correlation (+0.78, significant at 1% point) was also obtained between the weight of aftermath and the percentage of reducing sugars in the stubble on the day of uniform cutting. No correlations of significance were obtained between aftermath growth and any other carbohydrate fractions. The correlation coefficients between the weight of aftermath, and the percentages of nitrogenous constituents of the plants at the day of cutting were all negative and some were significant, such as, with the insoluble nitrogen of the removed tops (−0.77, significant at 1% point), with the total nitrogen of the removed tops (−0.65, significant at 1% point), and with the insoluble nitrogen of the stubble (−0.73, significant at 5% point). The obvious conclusion is that greater aftermath yields were obtained from larger plants, the larger plants being not particularly different from the smaller in available carbohydrates on a percentage basis but being somewhat lower in percentage of nitrogen, a common result with pot-grown plants. At 40 days after clipping, correlations in the same direction were

observed but none were significant. Certainly this limited evidence does not show that the production of aftermath is dependent very largely on the level of carbohydrate storage.

Summary

In continuation of studies on the reserve carbohydrates of orchard grass, plants were allowed to reach an early hay stage before cutting. Yields and carbohydrate and nitrogen analyses of tops, stubble, and roots were made before cutting for hay and at intervals up to 35 days after cutting. The yield of tops was closely related to the time of nitrogen application. Nitrogen fertilization 14 days before hay cutting did not alter significantly the dry weight yield but increased the protein content of the hay and decreased the sucrose and fructosan contents of the stubble and roots. The yield of aftermath was greatest when nitrogen was applied on the day when the hay was cut, but the percentage protein was highest when nitrogen was applied 14 days after hay harvest. Sucrose and fructosan were highest in the low nitrogen plants. Cutting of the hay modified the sucrose content of the stubble and roots in the pattern of the u-shaped curve previously described but did not change the fructosan in the same pattern. The application of nitrogen, whether applied on the day of cutting or 7 or 14 days later, decreased sucrose and fructosan of both stubble and roots as compared with those of unfertilized plants and increased total and especially soluble nitrogen in all parts.

A preliminary experiment to study the relation of carbohydrate reserves to the recovery of grasses is briefly described. Recovery as measured by the amount of aftermath growth was not related to the carbohydrate content of the reserve organs.

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BRIEF PAPERS

THE INTRACELLULAR DISTRIBUTION OF ASCORBIC ACID IN TURNIP LEAVES

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Whether ascorbic acid in leaf tissue is predominantly associated with chloroplasts or with the cytoplasm was discussed by RABINOWITCH (**8**), who summarized the results of numerous investigators but came to no definite conclusions. Since uncertainty exists about the question, a further attempt was made to estimate the relative amounts of ascorbic acid associated with cytoplasm and the chloroplasts.

Turnip leaves were selected for uniformity from plants growing in sand cultures in the greenhouse. They were carefully washed with tap water and rinsed with distilled water to remove dust residues. After removal of the mid-rib, the tissue of 15 to 25 leaves was cut into pieces 1 by 1.5 centimeters square. The pieces were gently but thoroughly mixed and wrapped in clean cheesecloth wet with distilled water. Duplicate samples of 1.5 or 2.0 grams were weighed out rapidly to a hundredth of a gram. One of the duplicates was blended for one minute in 100 ml. of 3% metaphosphoric acid in a Waring Blendor. The suspension was filtered through no. 2 Whatman filter paper, and an aliquot of the filtrate was used for ascorbic acid determination.

The remaining duplicate was blended for one minute in a chilled cup in 75 ml. of 0.5 molar sucrose and 25 gm. of frozen distilled water. During this operation, nitrogen gas was passed through the slurry. The suspension was filtered into chilled centrifuge tubes through glass wool and centrifuged at 3200 r.p.m. for 10 minutes on a centrifuge head with a radius to the bottom of the cup of 19.5 centimeters. The supernatant liquid was then siphoned off by suction in three portions: the first two were measured 35-ml. volumes; the first from the top and the second from the next lower portion of the supernatant liquid contained a clear solution. The third portion, comprising the rest of the fluid and the chloroplasts and cell fragments was taken from the bottom of the centrifuge tubes and measured in a graduate cylinder. The centrifuged chloroplasts and cell fragments were resuspended on this portion. The three portions were simultaneously poured into equal volumes of 6% metaphosphoric acid solutions in order to check the oxidation of the ascorbic acid. According to the work of NELSON and SOMERS (**7**), ascorbic acid is relatively stable in 3% metaphosphoric acid solutions. These suspensions were then filtered through no. 2 Whatman filter paper and aliquots

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were taken for ascorbic acid determination by the indophenol-xylene method of NELSON and SOMERS (**7**). The results were checked by the titration method with 2,6-dichlorophenol indophenol (**1**).

The results, presented in table I, show no great differences in the ascorbic acid values of the three portions of extract obtained with the sample ground in sugar solution and centrifuged. The concentration of ascorbic acid was, within the limit of error, the same in the portion containing the chloroplasts and associated residue as in the supernatant liquid. The percentage recovery (table I) shows some loss in ascorbic acid but this was not great enough to affect the general conclusion that ascorbic acid is not present in the chloroplasts at any appreciably higher concentration than in the other portions of the cell. The results do not provide evidence as to whether ascorbic acid

TABLE I

ASCORBIC ACID IN VARIOUS EXTRACTS CALCULATED AS MILLIGRAMS PER 100 GRAMS OF FRESH LEAF TISSUE.

Date	Sample	3% HPO_3 extraction	Sucrose—ice extraction based on amount of vitamin C in extract			Per cent. vitamin C obtained in sucrose extraction compared with 3% HPO_3 extraction
			Top layer	Middle layer	Bottom layer	
June 22	7	252	208	212	214	83.7
	8	240	196	200	189	81.2
	9	234	197	198	201	85.0
June 24	10	270	243	249	262	92.9
	11	268	204	208	215	78.0
	12	276	230	229	270	88.0
July 7	13	236	199	195	194	83.1
	14	240	220	216	223	91.7
	15	239	193	202	194	82.0
	16	250	175	178	184	71.6
	17	242	232	233	228	95.5

is excluded from the chloroplasts (*i.e.*, present in low or negligible concentration in the chloroplasts). The work of GRANICK (**2**), NEISH (**5**), and HILL (**3**) indicated that the above method of extraction does little physical damage to the chloroplasts. It seems likely, therefore, that if ascorbic acid is present in the chloroplasts at high concentration, the sample which contained the chloroplasts would have shown higher values than the other two samples.

In general, these results agree with the findings of WEIER (**9**) who found the reduction of the 2% silver nitrate, used in treating leaf strips of exposed cell tissue, took place in the cytoplasm predominantly rather than in the chloroplasts. These results confirm those of NEISH (**6**), who made titrations of ascorbic acid in leaf tissue, and found the quantity contained in the chloroplasts insignificantly different from the rest of the leaf tissue.

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ABSENCE OF ACID PHOSPHATASE FROM CHLOROPLASTS OF SPINACH AND IRIS[1]

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The presence of phosphatases in leaves is well known. The acid phosphatase of leaves appears to lack specificity with respect to hydrolyzable substrates (**1, 6, 7, 8**). Glycerophosphate has always been found to be an effective substrate (**1, 5, 6, 7, 8**). However, there is some evidence for the plurality of the acid phosphatase of leaves in that specific effects of activators and inhibitors have been found (**1**).

The objective of the present study was to determine whether or not chloroplasts contain acid phosphatase. This investigation was prompted by YIN's (**8**) inability to discern whether most of the acid phosphatase of the leaves of Iris and Lamium was concentrated in the chloroplasts themselves or in the surrounding cytoplasm at the interface with the chloroplasts.

Freehand sections of leaves of Iris and market spinach, and fractionated macerates of spinach leaves were tested for acid phosphatase by means of Gomori's microchemical method (**3**). All samples were washed with 0.2% versene and with several changes of distilled water, then incubated for periods of up to three hours at 37° C in the medium containing the substrate α-glycerophosphate at pH 5.1.

Almost no phosphatase activity was found in the freehand sections of Iris leaves. Less than 1% of the chlorenchyma cells showed activity, and this activity did not appear to be associated with the chloroplasts. In sections of spinach leaves the phosphatase was active in irregular patches of chlorenchyma. The precipitated lead sulphide was seen to occur in the extra-chloroplastic cytoplasm and at nuclei. There was very high activity on the crystals (druses) which occurred regularly in enlarged cells in the mesophyll. As was observed by GLICK and FISCHER (**2**), some cell walls, especially those of tracheids and epidermal cells, gave a positive reaction. However, this reaction was not the result of enzymatic activity since it also appeared in sections that had been boiled for three minutes.

Spinach leaves were macerated with a Waring Blendor in 0.2% versene. After filtration through muslin, the mixture was centrifuged at 6000 × g. for 15 minutes. The green sediment (fraction I), containing the chloroplasts and various unidentified particles, was resuspended in distilled water. The supernatant (fraction II) was light yellow in color. Both fractions were saturated with ammonium sulphate and the resulting precipitates were

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centrifuged out, resuspended in the incubating medium and left for three hours at 37° C with occasional stirring. Upon completion of the subsequent series of treatments, both fractions, but especially fraction II, showed the results of phosphatase activity. Fraction I, after being washed up to nine times prior to incubation, showed phosphatase activity. The same results were obtained when the material was embedded in collodion on microscope slides and dipped into the successive solutions, as when the material was freely suspended in each solution. However, examinations with both a light microscope and an electron microscope gave no evidence for the presence of lead sulphide within a plastid. The densities of both the grana and stroma remained unchanged. Crystals of lead sulphide could be seen adhering to the surfaces of a few of the plastids which also tended to cohere after centrifugation. The former were not the spurious effect of adsorbed lead (**4**) as they were not evident in the thermally inactivated samples.

The leaves of Iris and market spinach which were tested here did not contain acid phosphatase in their chloroplasts.

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SYMPTOMS OF MOLYBDENUM DEFICIENCY IN TOBACCO

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No report on the symptoms of molybdenum deficiency in tobacco has appeared in the literature. It seemed desirable, therefore, to determine and describe their nature since the information should be of value in mineral nutrition studies with tobacco.

Production of molybdenum deficiency symptoms in Connecticut Broadleaf tobacco was accomplished in water cultures purified by either a modified calcium carbonate or 8-hydroxyquinoline (oxine) method. The type of equipment and fixed-charge technique has been described (**4**). The few changes made for micronutrient studies included use of nutrient solution purification, pure micronutrients, and water condensed from greenhouse heating steam in Pyrex glassware. Pyrex plates were also substituted for the masonite pressboard covers of the 2.5-gallon culture jars.

The nutrient solution employed in the calcium carbonate purification experiments had the following composition:

A.		B.	
Water	600.0 ml.	Water	750.0 ml.
$Ca(NO_3)_2 \cdot 4\,H_2O$	300.0 gm.	K_2HPO_4	75.0 gm.
$Mg(NO_3)_2 \cdot 6\,H_2O$	70.0 gm.	$(NH_4)_2SO_4$	17.5 gm.
K_2HPO_4	9.0 gm.	$NH_4H_2PO_4$	6.0 gm.
Na_2S (saturated)	2 drops	Na_2S (saturated)	2 drops
$CaCO_3$	9.0 gm.	$CaCO_3$	6.0 gm.

These partial solutions were heated for one hour at 15 pounds pressure, shaken thoroughly, and let stand overnight before filtering. It was necessary to add the calcium carbonate to solution A in two portions, heating each time in order to approach neutrality. In one run, for example, the final pH of solution A was 7.12 and that of solution B was 6.97. Gaseous ammonia was liberated from solution B during treatment. The filtered solutions were each diluted to one liter. Five milliliters of each were added for every 990 ml. of water in the culture jars. A very heavy precipitate was formed on mixing.

The method followed in purification with oxine was largely that of HEWITT and JONES (**3**). Solution A had the following composition: Water, 500.0 ml.; $Ca(NO_3)_2 \cdot 4\,H_2O$, 150.0 gm.; $Mg(NO_3)_2 \cdot 6\,H_2O$, 35.0 gm. Solution B had the following composition: Water, 500.0 ml.; KH_2PO_4, 30.0 gm.; $(NH_4)_2SO_2$, 10.0 gm.

Each was acidified to pH 1.5 with concentrated hydrochloric acid, 60 mg. of iron as ferric chloride were added, then 24 ml. of oxine solution, and finally one normal KOH gradually with stirring to pH 5.0. The oxine solution consisted of one gram of oxine dissolved in 2.4 ml. of glacial acetic acid

and diluted to 80 ml. The quantity of oxine added was insufficient to remove all iron. The heavy black precipitate was filtered off after standing overnight and each solution diluted to one liter. Chloroform extraction of residual oxine was omitted. Ten milliliters of each solution together with 0.4 ml. of 1 *N* of K_2CO_3 were added for every 980 ml. of water in the culture jars. A slight precipitate was formed on mixing.

The micronutrient salts were the purest chlorides available and boric acid. The concentrations employed were Fe, 10.0; Zn, 0.20; Cu, 0.05; Mn, 0.65; B, 0.35, and Mo, 0.05 p.p.m. These quantities were sufficient to produce plants weighing about 60 gm. (air-dried) at flowering during fall and weighing about 150 gm. during spring. Plants grown in the spring usually displayed slight symptoms of iron and nitrogen deficiency if harvest was delayed too long after flowering. Initial acidities of the carbonate purified controls after addition of micronutrients approximated pH 5.8; and those of oxine purified controls were about pH 5.4. Final acidities at harvest were about pH 6.5 and 7.0, respectively.

The first indication of molybdenum deficiency was a savoying and mottling of lamina in midleaves about 24 days after the seedlings were placed in the solutions. Bending and twisting of the leaf lamina were usually present and were followed by the appearance of small interveinal necrotic areas that gradually enlarged until the entire leaf was withered. Leaf withering was frequently marginal at the start. These symptoms gradually spread to other leaves until almost all became involved. The molybdenum deficiency achieved was insufficient to cause more than a week of delay in blossoming and an approximately 35% loss in total dry weight. Whereas height of controls averaged 70 inches, that of a deficient plant was about 55 inches. These symptoms were quite different from those of any of the other micronutrient deficiencies (Fe, Zn, Cu, Mn, B) produced in plants grown at the same time. These results were obtained repeatedly in triplicate on omission of molybdenum.

The symptoms of molybdenum deficiency, like those of zinc and copper first appeared on the middle leaves of the plant, and only later on the upper and lower leaves. As was also the case with copper, the dead leaf areas became almost white. In zinc deficiency the dead areas were light to dark brown with dark irregular rings and granules. Figure 1 shows an advanced stage of moderate molybdenum deficiency in tobacco. Almost all the leaves were involved, the symptoms including crinkling, twisting, spotting and withering. The molybdenum content in the leaves of a control plant was determined by Mr. Glen Edgington of this station to be about 6.4 p.p.m. when supplied with 10 liters of solution containing 0.05 p.p.m. molybdenum. The concentration in leaves of a deficient plant of the savoy stage was slightly less than 0.3 p.p.m. This value is about six tenths of that suggested as a probable upper limit of the deficiency range in crop plants by Stout *et al.* (**5**).

Another check on molybdenum deficiency as the actual cause of these symptoms consisted in the response of cauliflower plants (Early Snowball)

when grown in the same solutions. Temperatures were too high to permit formation of heads in any of the plants. However, those plants from which molybdenum was withheld displayed severe symptoms of whiptail similar to those described by CLAYTON (**2**) and WALLACE (**6**). These consisted in formation of narrow, ruffled leaves with irregular margins, followed still later by leaves with vestiges only of lamina and swollen, tightly compacted bud leaves. Whiptail of cauliflower was found by Clayton to be widespread on unlimed soils of Long Island. AGARWALA (**1**) found it was necessary to

FIG. 1. Symptoms of molybdenum deficiency in Connecticut Broadleaf tobacco.

decrease supply of molybdenum to a maximum of 0.00005 to 0.0005 p.p.m., or less, in order to produce extreme symptoms of whiptail.

Through the aid of Dr. E. M. Roller, molybednum deficiency symptoms in tobacco were found to be associated with high nitrate and low coagulable nitrogen (protein) in the leaves. Soluble organic nitrogen (amino acids) increased only slightly compared to other micronutrient deficiencies.

Cauliflower appeared to require more molybdenum than tobacco. Leaves without lamina are evidence of extreme deficiency in cauliflower. The same level of molybdenum supply (*i.e.*, impurities) produced only moderate symptoms of deficiency in tobacco. On the basis of values determined by Agarwala and the responses obtained in these experiments with both plants, it

can be estimated that prevention of flowering in tobacco would require that molybdenum supply be decreased to 0.02 to 0.2 parts per billion, or 0.2 to 2.0 micrograms per plant.

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THE USE OF SEEDS WITH LOW CONTENT OF TRACE ELEMENTS IN STUDIES ON ESSENTIALITY OF MICRO-NUTRIENTS

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A recent contribution by Meagher *et al.* (**2**) has pointed out that there is a relatively large accumulation of molybdenum in normal seeds, and as a consequence, it is necessary to produce a special crop of seeds low in molybdenum in order to demonstrate molybdenum deficiency in the growing plant. Similar observations regarding other trace elements in seeds were made at the Biochemical Institute in Helsinki in the 1930's. It was emphasized, in connection with these observations, that it is important to produce seeds relatively low in trace elements in order to demonstrate the effect of micronutrient deficiency in experimental plants. Since these results are largely unknown they will be reviewed in brief.

In 1940 Virtanen and v. Hausen (**3**) reported on a series of experiments carried out over several years in order to find out how the organic substances present in the soil affect the growth of the pea during several generations. The uninoculated pea plant was grown in a 1.5-liter Erlenmeyer flask in sterile culture system in the nutrient solution of Hiltner (made up with tap water), with $Ca(NO_3)_2$ as the nitrogen source. The volume of the nutrient solution was 1.5 liters with a pH 6.1 to 6.7. The variety of pea was Torsdag (Svalöf). Two parallel series were grown. In series A the above-mentioned nutrient solution was used without any additions; in series B 200 gm. of air-dried garden soil was added to each flask. During the first experimental period the growth was good in both series. The next period, when seeds from the plants of the first experimental period were used, gave a quite different result. In series A the growth of the pea was poor and no seeds developed, whereas in series B the growth was good. Even during the second experimental period a good growth could be induced in the series A when the nutrient solution was supplemented with the ash obtained from the burning of the garden soil used for series B. The growth remained good throughout six successive experimental periods between 1935–1937; in each case the seeds obtained were used for the following experimental period. The results revealed that the normal seeds produced in agriculture obviously contained mineral substances which made relatively good growth of plants possible in nutrient solutions not containing the necessary trace elements in sufficient quantities to ensure normal growth. Only by growing plants in nutrient solutions deficient of trace elements was it possible to produce seeds which themselves were low in trace elements. Such seeds responded sharply to the lack of trace elements in nutrient solutions.

This paper did not state which particular trace element was lacking in the seeds, but a collaborator, ERKAMA (**1**), dealt with the problem in detail in his doctoral thesis in 1947. He showed that an addition of copper was sufficient to produce seeds on peas grown in the conditions described above (tap water without addition of trace elements). According to him, the copper content of the commercial seeds of the pea used in the experiments (Torsdag II) was 9.6 μg. per gm. of dry substance (about 2 μg. per seed), while in the seeds with low trace elements that were produced in tap water without addition of copper he found about 5 μg. of copper per gm. The seeds produced under a greater copper deficiency contained only 3 μg. of copper per gm., but in general they no longer germinated. On the basis of Erkama's results, it was evident that the copper deficiency in the nutrient solution used was one reason for the poor growth of pea in our previous experiments. There may also have been a deficiency of some other trace elements, but apparently the lack of copper was decisive.

The paper by MEAGHER *et al.* (**2**) gives a clear-cut picture of the necessity of producing low molybdenum seeds to demonstrate the need of this element. Since the findings in this laboratory have shown that the situation is the same in regard to copper, it is very possible that the same will also apply to some other trace elements. The content of trace elements in the seeds used for experimental plants may be an important consideration in studies on the essentiality and metabolic role of several of the micronutrients.

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PARTIAL SEPARATION OF THE PLASTID PIGMENTS BY PAPER CHROMATOGRAPHY[1]

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A method for separation of small quantities of plastid pigments was desired, and paper chromatography seemed to be the best prospect among the available techniques. A search of the literature revealed only one direct mention of the application of paper chromatography to the separation of the plastid pigments. STRAIN (**3**), in a review article, stated that chlorophylls and xanthophylls can be separated by paper chromatography using aqueous ethanol as the developing solvent. In a private communication Dr. S. Aronoff has informed us that he obtained a separation of the plastid pigments by repeated development of the paper chromatogram in Skelly-B (petroleum ether). When tested in this laboratory neither of these two methods gave a complete separation of all visibly present fractions.[2]

In these investigations plastid pigments were extracted from soybean and other leaves with acetone in a Waring Blendor and transferred to Skelly-B in a separatory funnel. The Skelly-B solution was washed thoroughly with water to remove acetone and acetone-soluble impurities, then concentrated under vacuum. One tenth of a milliliter of the Skelly-B solution was placed at the origin (fig. 1) on a square of Whatman no. 1 filter paper (23 cm. × 23 cm.) which previously had been washed with Skelly-B and air dried. The filter paper was stapled together to form a cylinder which was placed in a tightly covered widemouth gallon jar containing ca. 100 ml. of developing solvent. The paper was air dried after development in each of the successive solvents.

Among the solvents and solvent combinations tested the following sequence gave the best results:

FIRST DIMENSION.—(a) Acetone: All the pigments moved with the solvent front and the development was stopped when the solvent reached the top of the original drop. This procedure served to collect the pigments from the original circular spot in a line front from which they could be more easily separated in the later steps. (b) Skelly-B: The carotenes followed

[1] Journal paper no. J-2128 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project no. 1139.

[2] A paper by Bauer (BAUER, L. Trennung der Karotinoide und Chlorophylle mit Hilfe der Papierchromatographie. Naturwiss. 39(4): 88. 1952.) became available after this paper was submitted for publication. Bauer separated the plastid pigments into nine fractions by two-dimensional paper chromatography, using petroleum solvents which are not readily available in this country.

the solvent front while the other visible pigments moved only slightly, if at all, from the origin. The development was stopped when the solvent was ca. 20 cm. from the bottom of the paper. This step brought the carotenes so far ahead of the other pigments that they did not interfere with further separations. (e) One volume per cent. n-propanol in Skelly-B: All pigments moved, but this treatment separated the remaining pigments into the following groups (top to bottom): Chlorophyll *a* overlapping the xanthophylls, chlorophyll *b*, and an unknown yellow pigment which appeared below and well separated from chlorophyll *b* in the chromatogram. The development

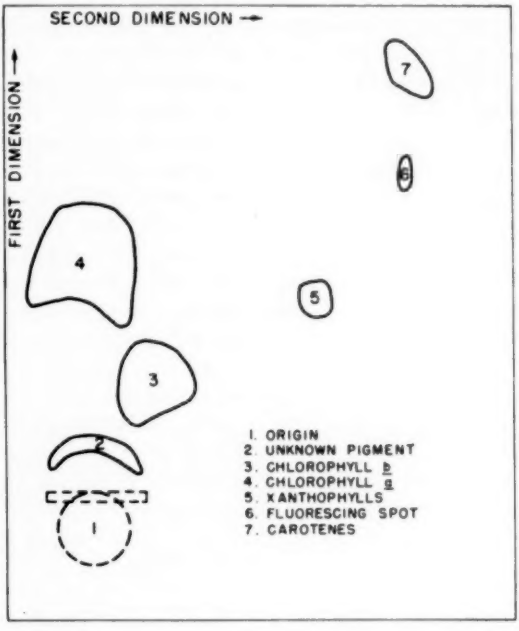

FIG. 1. Tracing of a paper chromatogram of soybean leaf pigments developed as described in the text.

was stopped when the solvent reached ca. 20 cm. from the bottom of the paper.

SECOND DIMENSION.—(d) Twenty-five volume per cent. chloroform in Skelly-B (the chloroform was thoroughly washed with water and dried with $CaCl_2$): The carotenes moved with the solvent front, the xanthophylls moved out of the chlorophyll *a*, which was almost stationary. Chlorophyll *b* moved, although much slower than the xanthophylls, and the unknown pigment did not move visibly. The development was stopped when the solvent had moved up ca. 16 cm.

After the completion of this development the chromatogram showed a distinct separation of the plastid pigments into six fractions: The carotenes, the xanthophylls, chlorophyll *a*, chlorophyll *b*, an unknown pigment, and a colorless but fluorescing compound found between the locations of the carotenes and the xanthophylls (fig. 1).

The identification of the unknown pigment was attempted by determining its absorption spectrum, which is shown in figure 2 together with the absorption spectrum of chlorophyll *b*. Absorption spectra of the carotene fractions and of chlorophyll *a* were also determined. The pigments were purified by the chromatographic technique described, redissolved in methanol and the absorption spectra measured with a Beckman spectrophotometer (Model DU).

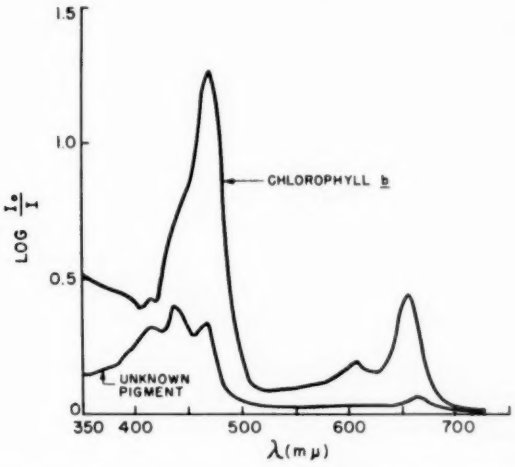

FIG. 2. Absorption spectra of chlorophyll *b* and the unknown pigment (neoxanthin?) dissolved in methanol.

The chlorophyll spectra conformed very closely to those reported by STRAIN and MANNING (**2**), and the absorption spectrum of the carotene fraction indicated that β-carotene was the main component. The unknown pigment had pronounced absorption maxima at 415, 437, and 467 mμ and a secondary maximum (fig. 2) at 662 mμ. The first three maxima indicate a carotenoid while the maximum at 662 mμ probably is due to impurities, possibly decomposition products of the chlorophylls. The unknown pigment may be neoxanthin, which was reported by STRAIN (**1**) to have absorption maxima at 434 and 464 mμ when dissolved in methanol and at 415, 437, and 467 mμ when dissolved in ethanol.

The method as outlined is a rapid qualitative method and it has definite possibilities of being made quantitative. Also, if some attention is given to

the details, the procedure makes a striking class-room demonstration. If one paper is started through the first dimension while a second, previously prepared, is started through the second dimension, a demonstration of the first and final steps can be completed in about one hour.

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THE DISTRIBUTION OF C^{14} IN THE ROOT AND SHOOT APICES OF YOUNG CORN PLANTS

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Received June 20, 1952

Concentration of P^{32} in the meristematic regions of plants has been reported by a number of investigators (**3, 5, 6**). RABIDEAU and BURR (**5**) have given evidence for a gradient in concentration of C^{13}-labeled compounds in the stem tips of bean plants. BELKENGREN (**2**) showed rapid movement of carbon-13-labeled materials to bean root tips after exposure of the leaves to $C^{13}O_2$ and light. GUSTAFSSON *et al.* (**4**) demonstrated accumulation of P^{32} in the meristematic regions of barley roots. These findings and other considerations have emphasized the desirability of having more information concerning the movement of carbon from leaf-fed carbon dioxide into the meristematic regions of both shoots and roots. In relation to many questions pertaining to growth and development it would be desirable to have information not only with respect to the concentration of carbon in meristematic regions but also with respect to the incorporation of labeled carbon into specific compounds and the distribution of these compounds in the growing regions.

The present study was designed to yield information on the concentration of C^{14} in meristematic regions, the nature of the C^{14}-labeled fractions, and the distribution of these fractions in root and shoot apices of young corn plants.

Seedlings of an inbred line of corn, University of Texas number 859, were grown in sand in a greenhouse until they were three to five inches tall (8 to 10 days). They were then transferred to a black lucite disk which served as a plant holder (fig. 1). This holder was perforated with 15 holes in which two-inch lengths of glass tubing were cemented to serve as plant supports. The plants were held in these glass tube supports with a cotton plug in the lower part of the tube. The holder was placed over a desiccator bottom so that the roots of the seedlings dipped into a Shive nutrient solution. This solution was aerated continuously and black cloth was tied around the desiccator as a light screen.

The plants were grown in this container for five days. At the end of the fifth day the plants were sealed in the glass tube holders by pouring melted beeswax on top of each cotton plug. When poured at 50° C, this wax caused no detectable damage to the plants. A bell jar of 10 liters volume was placed over the leaves of the plants and sealed to the lucite plant holder with grease. It was held tightly against the lucite disk with several steel springs.

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Approximately 100 microcuries of C^{14} (as carbon dioxide) were introduced into the bell jar through a side port. The leaves were illuminated for a 24-hour period with a single 300-watt tungsten photoflood lamp placed 30 inches above the leaves. The daylight portion of the 24-hour period was of approximately 12 hours duration.

During the illumination period, root aeration was continued. The pressure resulting from aeration effectively prevented the entrance of $C^{14}O_2$ from the upper chamber. Alkali absorption towers were used to absorb CO_2 in both the entrance and exit air lines. At the close of the illumination period the residual CO_2 in the bell jar was passed through a third alkali tower. The weights and radioactivity analyses of carbonate precipitates recovered from these towers were used to calculate the radioactivity absorption.

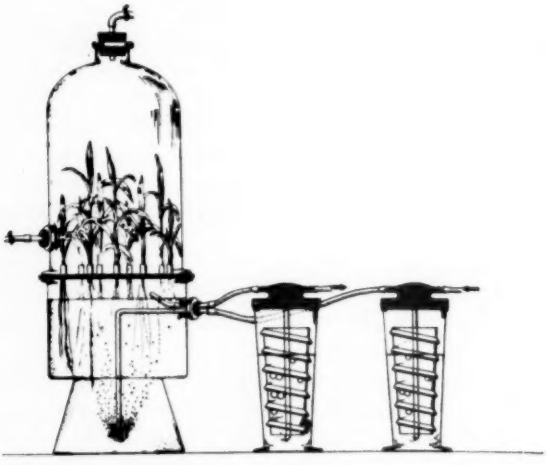

FIG. 1. Diagram of the chamber in which young corn plants were grown and their leaves exposed to light and to $C^{14}O_2$. See text for description.

After the exposure period, the apparatus was disassembled and the plants were removed. Root apices (both terminal and lateral) and shoot apices were harvested from each plant. Root apices consisted of the terminal portions 3 mm. in length while stem apices consisted of terminal portions 2 mm. in length. A few root apex samples were taken for micro-autoradiographic study. The remainder of the root apices were collected and combined for fractionation and radioactivity analyses. All shoot apices were combined for similar fractionation and radioactivity measurements. The remaining portions of the plant were likewise combined and treated similarly. Samples were boiled in 80% ethyl alcohol and the alcohol extracted with ether. The fractionation followed standard procedures (**1**). Fractions separated were the ether-soluble, residual alcohol extract, principal nitrogenous, acid hydrolysate and crude fiber.

Because of varying lengths of extraction times some of the fractions

obtained by this procedure were mixtures. Fraction 1 (ether-soluble) consisted largely of chlorophyll and carotenoid pigments and a small quantity of fat; fraction 2 (residual alcohol extract) was composed of approximately equal quantities of sugars and organic acids, with a small quantity of amino acids; fraction 3 (principal nitrogenous) consisted mostly of protein hydrolysate; fraction 4 (acid hydrolysate) was principally starch hydrolysate; and fraction 5 (crude fiber) was unpurified cellulose residue.

The fractions were placed in sample cups and the solvents evaporated. The samples were then weighed and their radioactivity values determined with a Geiger-Muller counter. Although radioactivity measurements were corrected for background, inasmuch as the geometry of sample measurement

TABLE I

WEIGHTS AND RADIOACTIVITY MEASUREMENTS OF THE FRACTIONS SEPARATED FROM VARIOUS PARTS OF CORN PLANTS WHICH WERE EXPOSED TO C^{14}-LABELED CARBON DIOXIDE AND LIGHT.

Parts of plant	Fraction	Weight	Radioactivity	
		mg.	*counts/min.*	*counts/min./mg.*
Root apices	Ether soluble	14	400	29
	Residual alcohol extract	18	1911	106
	Principal nitrogenous	6	228	38
	Acid hydrolysate	336	1170	4
	Crude fiber	2	26	13
Shoot apex	Ether soluble	24	64	3
	Residual alcohol extract	18	1072	61
	Principal nitrogenous	17	51	3
	Acid hydrolysate	349	276	1
	Crude fiber	2	3	3
Remainder	Ether soluble	102	6525	65
	Residual alcohol extract	70	8759	125
	Principal nitrogenous	62	704	12
	Acid hydrolysate	607	3484	6
	Crude fiber	25	1953	80

was constant and sample thickness was uniform, they were not corrected for deviations resulting from geometry or self-absorption.

Micro-autoradiograms were made from paraffin-embedded root apices (sectioned longitudinally at 8 to 10 microns) using standard histological procedures. Paraffin ribbons were floated on warm water (40° C) for straightening, transferred to cool water and then floated onto microscopic slides covered with photographic film (Eastman nuclear track plates, NTB–2, 10 micron emulsion thickness). All operations involving the use of nuclear track plates before photographic development and fixation were carried out in darkness. Plates were placed in a light-tight lead-lined box for a five to eight day exposure period. At the end of this exposure period, paraffin was removed from the sections with xylene, and the slides were hydrated through a standard series of alcohol concentrations to water. After a five-minute period in running water, the slides were subjected to photographic develop-

ment. Following photographic fixation, the slides were washed, and the sections stained, to aid in microscopic observation, with Delafield's hematoxylin. The slides were then dehydrated with a graded alcohol series, and mounted in clarite for examination. No radioautographs were made of shoot apices.

Weights and radioactivity measurements of fractions are shown in table I. Calculations show an absorption of 8.5 microcuries of radioactivity in this experiment. The figures for this single set of determinations are typical of the three sets of determinations which were made. Because there were minor variations in the amounts of C^{14} used and in the exposure period, the results

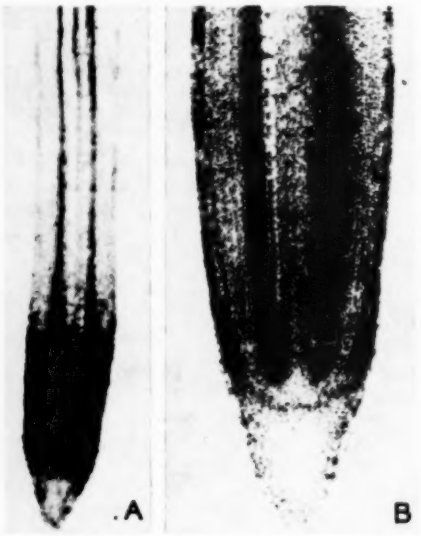

FIG. 2. Autoradiograms of longitudinal sections of young corn root apices showing the distribution of C^{14} after exposure of the leaves of the plant to light and $C^{14}O_2$. A. Approximate portion of a root used for fractionation procedure (×50). B. Median longitudinal section of the terminal millimeter of a root showing the relative distribution of radioactivity (×80).

of these three sets were not averaged. Although the absolute radioactivity values of the various fractions differed slightly, the values were relatively the same in all three experiments.

The greatest radioactivity, both total and specific, was in compounds extracted by 80% alcohol. While considerable total radioactivity was found in the acid hydrolysate fraction, this fraction was, compared with the other fractions, so large that its specific radioactivity was quite low. The radioactivity values of the ether-soluble fraction and principal nitrogenous fraction were intermediate in value, whereas the crude fiber fraction was lowest with regard to both weight and radioactivity values. The shoot apex samples generally had lower radioactivity values than the root apex samples.

The radioactivity values of fractions from both shoot and root apices were generally lower than fractions from the remainder of the same plants. The relationship among the shoot apex fractions themselves was similar to the relationship among the fractions of the root apex samples.

The stem apex and the root apex are regions of active growth and/or metabolism, hence translocation of considerable radioactive material to these regions is to be expected. Movement of labeled materials to such regions has been demonstrated previously (**2, 3, 4, 5, 6**). Although it is possible that C^{14} might have entered the stem apex from the gas phase, as well as by translocation of labeled materials, the C^{14} found in the root apices arrived there solely as a result of translocation. By employing the isolation technique used by BELKENGREN (**2**) it was determined that only an infinitesimally small amount ($2 \times 10^{-5}\%$) of the total radioactivity appeared in the constantly renewed air phase in the root chamber. A comparable amount was found in the nutrient solution at the close of the experiment. There was thus little possibility of air transfer of C^{14} to roots during the C^{14} exposure period.

Examination of the autoradiograms (fig. 2) reveals that the greatest amount of radioactivity was found in the terminal millimeter of the root apex. Radioactivity was most concentrated in the inner cortex, outer stele and in the superficial cells. Radioactivity was less evident in the more basipetal portions of the longitudinal sections, although it was again most concentrated in the stelar regions and the superficial cells. The dark areas of the more basipetal portions of the root apices may indicate the path of translocation of C^{14}-labeled compounds.

This work was supported in part by the Clayton Foundation for Research.

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WATER-COOLED LAMP SYSTEMS WITH REFLUXING AQUEOUS FILTERS[1]

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When plants are grown under high irradiances of incandescent lamp radiation, temperature control becomes difficult unless the infrared is removed. The infrared energy radiated by standard incandescent lamps is from 75 to 85% of the input power (**2**). Excessive heating of exposed portions of the plant results from direct absorption of infrared energy since most of that energy is in the region of the water absorption bands.

The most effective filters for removing the near infrared are water or solutions of the ferrous or cupric salts (**3, 4, 5**). If the filters consist of shallow tanks open to the air, the rapid loss of water due to evaporation necessitates constant maintenance. Since most of the radiated energy is absorbed in the top layer of water, the temperature gradient does not induce appreciable convection. While cooling coils placed in the body of the liquid may keep the bottom of the tank cool, they do not appreciably reduce the evaporation rate.

In attempting to solve the problem of evaporation in unsealed systems, it was found that if the water-cooling coils were suspended in the air space above the water surface, the water vapor was condensed on the coils and refluxed back into the tank. In addition, the cold condensate dropping upon the water surface set up convection currents, further reducing the temperature and evaporation. If the coils are placed near the top of the unit around the lamps, the air temperature is kept within safe limits for a completely enclosed but not sealed system.

Two types of lamp unit which have been constructed and used extensively in our laboratories are shown in detail in figures 1 and 2. Both of these units are designed for use with a layer of water or solution and the standard line of internal reflector lamps. A 10-cm. layer of water, as used in the large lamp unit of figure 2, absorbs all infrared beyond ca. 1150 mμ (**1**). The addition of 30% ferrous ammonium sulphate decreases the limit to ca. 760 mμ and copper sulphate can be used to remove both red and infrared beyond ca. 600 mμ. The 75-watt incandescent internal reflector lamp is four inches in diameter, and the 150- and 300-watt incandescent and the 100-watt mercury arc lamps are five inches. The incandescent lamps are available as reflector flood or spot lamps. The spot lamp is especially useful for very high irradiances over a small area; the flood is better for irradiation of larger areas.

[1] Published by permission of the Secretary of the Smithsonian Institution.

In the construction of these lamp units, special consideration must be given to the selection of water-resistant and thermally stable materials. The lamp holders or sockets must be all porcelain. Porcelain sockets with plastic center insulation will eventually arc and fail. The top mounting plates should be of a water-resistant, resin-impregnated asbestos board such as is used for electrical insulation or chemical bench tops (Chemstone). Unimpregnated asbestos Portland cement panels may be used if properly treated with a heat-resistant enamel, preferably of the baking type.

The small unit of figure 1 has proved very useful for the general irradiation of single large plants or small cultures of seedlings or algae. It can be mounted on small cabinets in combination with gelatin or glass filters where narrow spectral regions are required. The glass vessel may be either a 4000-ml. standard beaker or a molded Pyrex cylinder six inches in diameter

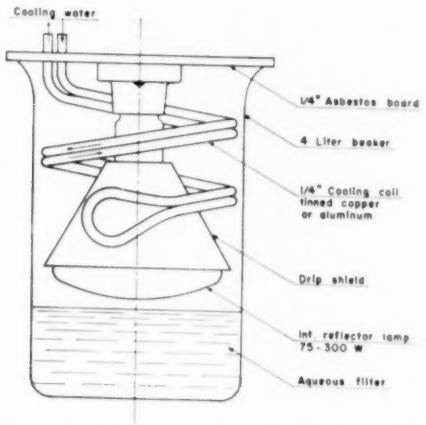

FIG. 1. Single water-cooled refluxing lamp unit.

and 12 inches high. Eight linear feet of tubing with an outside diameter of one fourth of an inch when wound into a double spiral is adequate for a 300-watt lamp when the cooling water runs below 20° C. Since copper tubing becomes oxidized with continued operation, it is desirable to use either tinned copper or aluminum. The coil can be made readily by bending the eight-foot length of tubing in the middle, after first annealing a foot of the center section to prevent collapse on bending. The two legs then can be soldered at various points along the length and coiled on a mandrel. The upper part of the coil should be supported by tying with copper wire or soldering to a pair of long machine screws bolted into the top (not shown in diagram).

The outdoor type of internal reflector lamp of hard glass will withstand contact with cold water without breaking, but these lamps are more expensive than the indoor type. The indoor type may be used if a drip shield of aluminum or copper is employed. These shields consist merely of a trun-

cated cone as shown in figure 1. Aluminum or copper foil wrapped about the upper part of the lamp will serve the same purpose.

A 300-watt lamp unit will deliver ca. 1200 fc at 12 inches; the radiant flux distribution is more uniform if the lamp system is used within a white enclosure. Using a beaker with a lip open to the atmosphere, the unit can be operated with a 300-watt lamp continuously for a period of several weeks with very little water loss. Without the cooling coil, such a system requires the addition of water several times daily.

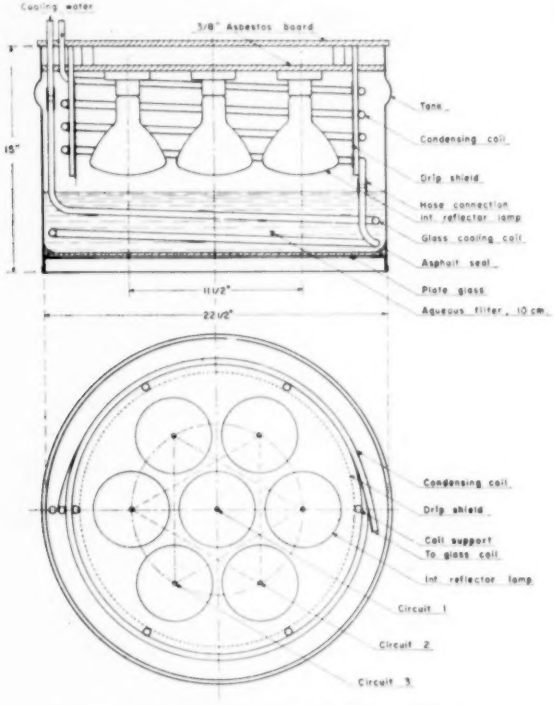

FIG 2. Multiple water-cooled refluxing lamp unit.

The multiple lamp unit (fig. 2) is designed to operate with a maximum power input of 2100 watts (seven 300-watt lamps). A cooling coil made of 25 feet of copper or aluminum tubing of an outer diameter of three eighths of an inch usually will keep all parts of the system cool, but 50 feet has been found more satisfactory when maximum power is used. Seven lamp holders are mounted as shown, six in a ring and one in the center. Three of the six in the ring are alternately wired to one outlet connector, the other three to a second, and the center lamp to a third, thus making it possible to operate the unit with one to seven lamps as desired. When three or fewer lamps are required for an experiment, six lamps can be installed, making it possible to

switch to an alternate set in case of lamp failure. The cover supporting the lamp holders consists of two panels, one lower centering panel within the tank, separated from the top panel by spacers. All the wiring is confined to the space between the two panels.

It was found that occasionally lamps broke because of water dripping from the upper coils onto the water surface, causing splashing upon the bottom face of the bulb. This was completely eliminated when copper screen wire was mounted as a cylinder on the inside of the coils. Solid sheet material could not be used since it interfered with air circulation.

In experimental work where precise air temperature control is required, it also is necessary to cool the aqueous filter layer directly. Where water alone is used, copper or aluminum tubing is quite satisfactory, but filters such as acidulated copper sulphate solutions attack all the common metals as copper, brass, aluminum, tin, and lead, and it is necessary to use tubing of stainless steel, silver, or glass. Rubber and plastic tubing do not have sufficient thermal conductance to be effective unless very large surfaces are employed. The coils may be held to the upper assembly by copper wire covered with vinyl plastic electrical sleeving.

The tanks can be constructed from standard 50-gallon alcohol drums; two tanks can be obtained from each drum. A 15-inch section is cut from either end of the drum, forming the two tanks. The bottom of each tank is then cut out so as to leave a one-inch flange to hold a glass bottom. The cutting of the drums is effected readily by a high-speed power-driven hack-saw mounted in a portable electric drill.

Where only water is used as the filter, vinyl synthetic resin air-drying finishes such as Tygon are fairly satisfactory for coating the inside of the tanks. However, all air-drying finishes which we have used failed in time and this failure was greatly accelerated by acidulated solutions. The only finish found satisfactory for such use is the baking-type of polymerizing vinyl resin such as used for coating industrial electroplating pickling tanks. One such finish which has proved especially satisfactory is white Micerosol (E-1266, Michigan Chrome and Chemical Company). The primer is brushed on and baked for 15 minutes at 180° C; then the adhesive brushed on and baked in a similar fashion. The final coats of Micerosol are applied as two or three layers by brushing, each coat given a short bake of 15 minutes at 180° C to just set the material. After all the coats have been applied, the tank is given a final bake at 180° C for two hours. The final finish is tough and rubbery and appears to be resistant to strong acids and alkalis and will withstand high temperatures.

The glass bottom is sealed into place with a high-melting air-blown asphalt compound similar to the type used for sealing storage batteries. Rubberseal no. 3 (Mitchel Rand Insulation Company) is very suitable and does not harden with age. It is applied by pouring a fillet of the hot asphalt on the bottom tank flange. The warmed glass is then gently lowered onto the asphalt and the whole assembly placed in an oven at 100° C until a

complete seal is effected. Aquarium cements are not suitable when acids are used.

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NOTES

Southern Section.—The Southern Section of the A.S.P.P. met with the Association of Southern Agricultural Workers in New Orleans, Louisiana, February 9–11, 1953. In addition to three sessions of submitted papers, a symposium was held jointly with the Forestry Section of the Association. Abstracts of all papers will appear in the 1953 Proceedings of the Association. Dr. C. H. Wadleigh gave an address entitled "The Efficiency of Water Use by Plants" at the traditional breakfast meeting. The breakfast and the meetings were well attended. Officers of the Section for 1943–54 are Chairman, Donald E. Davis, Alabama Polytechnic Institute; Vice-Chairman, Roger W. Bledsoe, Florida Agricultural Experiment Station; Secretary-Treasurer, John B. Whitney, Clemson Agricultural College; Directors, Paul J. Kramer, Duke University; E. M. Emmert, University of Kentucky; G. M. Shear, Virginia Agricultural Experiment Station; Howard B. Sprague, Texas Research Foundation.

Charles Walter Bacon.—The American Society of Plant Physiologists records with sorrow the passing of one of its members, Dr. Charles Walter Bacon, on March 19, 1953, at his home in Washington, D. C. Dr. Bacon, plant physiologist and chemist, who had carried on research in the United States Department of Agriculture on the improvement of tobacco for 42 years, had been ill since July 1952. He was widely known for his research in tobacco and had many technical and practical papers published. Dr. Bacon was born at Worcester, Massachusetts, in 1886. He received his Ph.D. degree at Clark University in chemistry. Dr. Bacon is survived by his wife, Janie Warren, and twin daughters, Mary and Ruth.

Physiology of Seeds.—W. Crocker and Lela V. Barton. Chronica Botanica Company, Waltham, Massachusetts. 267 pages, 1953. $6.50.

This volume is a valuable outline of present knowledge concerning seeds and germination. It is written from the viewpoint of the investigator, and it describes unsolved problems and aspects of seed physiology requiring reinvestigation as well as the established knowledge concerning seeds and germination. With each chapter there is a list of references to the scientific literature. The topics of the book are the anatomy of seeds, seed production, the chemical composition of seeds, the water relations of seeds, respiration of seeds, factors affecting germination, dormancy of seeds, storage and life span of seeds, the metabolic and energy changes in seed development and germination, vernalization, embryo culture, and seed transmission of disease. A subject index and an author index are included.

Cytochemistry: A Critical Approach.—J. F. Danielli. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, New York. 1953. $4.00.

This small volume consists of the material presented by the author in a series of lectures at the University of Chicago in 1949. It is not intended as a textbook but is an integrated attempt to introduce rigorous experimental criteria into the development of methods in cytochemistry. In the introduction a number of techniques are examined critically to illustrate the hazards which exist in their use. Other chapters describe fixation procedures, studies on alkaline phosphatase, the critical study of the cytochemistry of aldehydes, the cytochemistry of proteins and nucleic acids, quantitative studies in cytochemistry and the future outlook in cytochemistry. The book is illustrated with 12 plates of excellent photographs of cytochemical preparations and it has a subject index.

Plant Anatomy.—Katherine Esau. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, New York. 735 pages, 1953. $9.00.

This book is a critical and comprehensive analysis at the research level on the structure and organization of vascular plants. Brief, but adequate attention is given to the historical aspects, with primary emphasis upon more recent developments relative to cells, tissues and parts. The contributions of many workers are summarized and their papers are cited with a bibliography concluding each section. A valuable feature is the author's introduction to each section under the title "Concept" which briefly characterizes the given tissue or part, as well as views concerning it, thus stressing its individuality with less dependence upon formal definition. The author, who has contributed much to developmental plant anatomy, summarizes in an impersonal and scholarly way, the recent advances within this area. The descriptive value of the volume is greatly increased by the liberal use of excellent illustrations, including about 80 pages of special plates printed on heavier paper.

Soil Microbiology.—S. A. Waksman. John Wiley and Sons, Inc., 440 Fourth Avenue, New York 16, New York. 356 pages, 1952. $6.00.

Intended as a textbook, the purpose and scope of this volume, as stated in the preface, is "to survey the nature and abundance of microorganisms in the soil, to review the important role that they play in soil processes and . . . to show the relation between them and soil fertility." There are sixteen chapters, each with a selected bibliography. While emphasis is on bacterial transformations, the entire subject is reviewed and the references are sufficiently extensive to lead the interested student to sources of fuller information than can be given on the many topics treated, where mention must necessarily be brief when it is attempted to cover so vast a subject in relatively few pages.

Introductory Mycology.—C. J. Alexopoulos. John Wiley and Sons, Inc., 440 Fourth Avenue, New York 16, New York. 482 pages, 1952. $7.00.

This book provides a general survey of fungi, in the wide sense, intended to give students a reasonable knowledge of the group and to provide an adequate background for those who wish to undertake special studies in the field. A chapter on the bacteria, regarded by the author as not properly grouped with the fungi in the strict sense, and one on the slime molds, regarded as doubtfully related, are included for the sake of completeness. The treatment of the remaining groups is, in its broad outline, similar to that generally accepted at the present time. The illustrations, whether original or adapted, are in general excellent, and the numerous diagrammatic life-cycles are sure to be helpful. Bibliographies are appended to each chapter and are ample and carefully selected.

AMERICAN SOCIETY OF PLANT PHYSIOLOGISTS

Organized in 1924 for the advancement of Plant Physiology as a pure and applied field of botanical science

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Any one actively interested in Plant Physiology is eligible for membership in the Society. Candidates for membership must be recommended by two members, and approved by executive committee, or by the vote of the Society at some annual or special meeting.

The payment of the annual dues to the Society entitles the members to receive the official journal, Plant Physiology. They will receive in addition the regular bulletins and announcements sent out periodically by the secretary. Members are invited to take a personal interest in the growth and development of the Society.

Manuscripts for Plant Physiology should be addressed to Prof. Walter F. Loehwing, Department of Botany, State University of Iowa, Iowa City, Iowa. Drawings and photographs should not exceed 8.5 × 11 inches in size, should be numbered correctly on the back, and separate typewritten legends included with the text of the manuscript. Tables and cuts should be used sparingly, and the text presented in concise form. Instructions to authors for preparation of manuscripts are given in the January number, Volume 28, 1953, of Plant Physiology, 149–150.

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